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Regulation der Interaktion der präsynaptischen Vesikelproteine Synaptophysin und Synaptobrevin

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Zusammenfassung

Die integralen Vesikelmembranproteine Synaptophysin und Synaptobrevin interagieren in adulten Neuronen. Zusätzlich bildet Synaptobrevin mit den Plasmamembranproteinen Syntaxin und synaptosome-associated protein 25kDa (SNAP25) den SNAP-Rezeptor (SNARE)-Proteinkomplex, der Voraussetzung für die Fusion zwischen synaptischen Vesikeln und präsynaptischer Membran ist. Mit Synaptophysin interagierendes Synaptobrevin bindet jedoch nicht an den SNARE-Proteinen. Es wird daher vermutet, dass der Synaptophysin/Synaptobrevin-Komplex eine Art Reservepool für Synaptobrevin bei erhöhter neuronaler Aktivität darstellt und die Verfügbarkeit von Synaptobrevin während der Exozytose reguliert. Mit verschiedenen Ansätzen wurde versucht, den auf dem Vesikel befindlichen Komplex genauer zu charakterisieren und in seiner Funktion näher zu beschreiben.

Nach Stimulation mit exozytosevermittelnden Substanzen dissoziierte der Synaptophysin/Synaptobrevin-Komplex, sowohl unter nativen Bedingungen als auch bei Blockierung des finalen Fusionsereignisses. Dieser Prozess war calciumabhängig, konnte jedoch nicht durch die direkte Wirkung von Calcium ausgelöst werden. Die Untersuchung des Komplexes mit Hilfe von clostridialen Neurotoxinen zeigte, dass Synaptobrevin bevorzugt in Bindung an Synaptophysin und als Dimer gespalten wurde. Die Spaltung des SNARE-Proteins SNAP25 hatte keinen Einfluss auf die Komplexbildung. Die Verringerung des Cholesterolgehaltes der Membran führte zur Abnahme der Interaktion von Synaptophysin und Synaptobrevin, umgekehrt zeigte sich ein Anstieg bei zusätzlicher Cholesterolapplikation. In weiteren Experimenten konnte der C-terminale Teil des Synaptobrevins als für die Bindung zu Synaptophysin entscheidende Abschnitt identifiziert werden. Weiterhin konnte die erfolgreiche Translokation von rekombinanten Konstrukten aus Botulinumtoxin D und einem angekoppelten funktionstüchtigen Protein ins Zytosol gezeigt werden.

Synaptophysin besitzt in adulten Neuronen eine modulatorische Funktion auf die Bereitstellung von Synaptobrevin während der Exozytose. Die Komplexbildung der beiden Proteine wird dynamisch geregelt und korreliert mit der neuronalen Reifung.

Schlagwörter:

Synaptophysin, Synaptobrevin, Synaptophysin-Synaptobrevin-Komplex, Syp/Syb-Komplex, SNARE-Komplex, Exozytose, Vesikelfusion, neuronale Stimulation, clostridiale Neurotoxine, Botulinumtoxin, Cholesterol

Abstract

The vesicle associated membrane proteins synaptophysin and synaptobrevin interact in mature neurones. Additionally synaptobrevin forms a complex with the plasma membrane proteins syntaxin and synaptosome-associated protein 25kDa (SNAP25), better known as the SNAP-Receptor (SNARE) complex, which is a prerequisite for fusion of the presynaptic and vesicle membranes. These two protein complexes however are mutually exclusive. It is assumed that the synaptophysin/synaptobrevin complex resembles a reserve pool for synapto-

brevin and regulates the availability of synaptobrevin for the fusion process in case of increased synaptic activity. Different approaches where chosen to characterize this protein complex and to examine its function in more detail.

After excessive stimulation the synaptophysin/synaptobrevin complex dissociates, even when the final fusion process is blocked. This step was dependent on the presence of calcium, though it could not be triggered directly by calcium administration. When using clostridial neurotoxins, synaptobrevin was preferentially cleaved in its homodimeric form and in the complex with synaptophysin. Cleavage of SNAP25 had no effect on the complex formation. Depletion of cholesterol content decreases the interaction of synaptophysin with synaptobrevin, while cholesterol treatment increases interaction. Further experiments indicated that synaptophysin binds to the the carboxy-terminal transmembrane part of synaptobrevin. Furthermore it could be shown that proteins attached to botulinum toxin can be delivered to the cytosol of neuronal cells, being fully active.

In mature neurons synaptophysin plays a modulatory role, facilitating the interaction of synaptobrevin with its SNARE partners. The interaction of synaptophysin and synaptobrevin is dynamically controlled and correlates with synaptic maturation.

Keywords:

synaptophysin, synaptobrevin, synaptophysin-synaptobrevin complex, Syp/Syb complex, SNARE complex, exocytosis, vesicle fusion, neuronal stimulation, clostridial neurotoxins, botulinumtoxin, cholesterol

Abkürzungen

TeNt - Tetanustoxin; BoNT/X - Botulinumtoxin vom Serotyp X; scBoNT - single chain BoNT; HC - schwere Kette; LC - leichte Kette; DIV - days in vitro; IP - Immunpräzipitat; AK - Antikörper; AS - Aminosäuren; NSF - N-ethylmaleimide sensitive factor; SNAP25 - synaptosome-associated protein 25kDa; SNAP - soluble NSF attachment protein; SNARE - SNAP Rezeptor Komplex; β-MCD - β-Methylcyclodextrin; SDS–PAGE - Sodiumdodecylsulfat Polyacrylamid Gelelektrophorese; GFP - green fluorescent protein; His-Tag - 6 Histidin Markierung; C - Carboxy-terminal; N - Amino-terminal; ATP - Adenosintriphosphat; mRNA - messenger ribonucleic acid; IPTG - Isopropyl-β-D-Thiogalactopyranosid; Ni-NTA - Nickel Nitrilotriessigsäure

Inhaltsverzeichnis

1 Einleitung

Die calciumabhängige Exozytose von Vesikeln an der Synapse ist ein fundamentaler Prozess in der neuronalen Signalübertragung. Damit der Neurotransmitter in den synaptischen Spalt freigesetzt werden kann, müssen Vesikel gebildet und mit dem jeweiligen Molekül gefüllt werden, dann zur präsynaptischen Membran sortiert werden und dort andocken. In einem Zwischenschritt, der Priming genannt wird, werden die Vesikel auf die Calcium-abhängige Exozytose vorbereitet und fusionieren schließlich mit der präsynaptischen Membran.

Die Membranfusion ist ein unspezifischer Prozess, d.h. sie kann durch verschiedene Ursachen ausgelöst werden wie z.B. Druck, Elektroschocks oder Proteininteraktion. Es muss jedoch immer Energie aufgewendet werden, weshalb die Fusion nicht spontan abläuft. Die gesteuerte Fusion von Membranen spielt nicht nur eine Rolle bei der synaptischen Übertragung zwischen Neuronen und an der neuromuskulären Endplatte, sondern auch bei der Freisetzung von sekretorischen Vesikeln, intrazellulären Prozessen an Endosomen oder auch beim Eindringen behüllter Viren in eine Zelle. Die Proteine, die diesen Prozess steuern, scheinen evolutionär konserviert zu sein und finden sich in ähnlicher Art z.B. auch in Hefepilzen (Jahn und Sudhof, 1999).

Ein Schlüsselereignis in der Interaktion zwischen präsynaptischer und Vesikel-Membran ist die Formation des so genannten SNAP-Rezeptor-Protein-Komplexes (SNARE-Komplex), der aus dem Vesikel Assoziierten Membran-Protein 2 (VAMP2, Synaptobrevin) und den Plasmamembranproteinen SNAP25 und Syntaxin besteht (Sollner et al., 1993). Über deren Verdrillung werden die gedockten Vesikel in Position für die Exozytose gebracht (Chapman et al., 1994).

Den ersten Hinweis auf die Funktion der SNARE-Proteine gaben clostridiale Neurotoxine. Diese katalysieren abhängig vom Serotyp die spezifische Spaltung eines der drei SNARE-Proteine und bringen damit die Exozytose komplett zum Erliegen (Ahnert-Hilger und Bigalke, 1995). Die clostridialen Neurotoxine, zu denen neben den 7 Botulinumtoxinen auch das Tetanustoxin gehört, sind typische Zwei-Domänen Toxine. Die leichte Kette (light chain, LC) enthält die enzymatische Peptidase mit einem der SNARE-Proteine als spezifischem Substrat. Die schwere Kette (heavy chain, HC) ist für die Aufnahme des Toxins in das Neuron entscheidend. Diese Toxine werden schon lange als Werkzeug für die Untersuchung des synaptischen Exozytoseapparates genutzt (Schiavo et al., 1994). Deshalb ist es von großem Interesse, die Funktion der Toxine detailliert zu eruieren, einerseits um damit den Prozess der Vesikelexozytose genauer untersuchen zu können, andererseits zur Evaluation der Verwendbarkeit als spezifischen Transporter für zukünftige Pharmaka.

Mit dem SNARE-Komplex interagieren verschiedene Proteine, von denen bisher nur wenige genau charakterisiert werden konnten. Diese Proteine modulieren die Funktion des Komplexes und beeinflussen unter anderem seine Entstehung, die gezielte Wirkung und seine Wiederauflösung.

Das mit dem 18kDa schweren SNARE-Protein Synaptobrevin interagierende Synaptophysin ist eines der häufigsten Vesikelproteine und weist mit seinen 38kDa und 4 Transmembrandomänen starke antigene Eigenschaften auf. Synaptophysin wird deshalb bereits seit langem als vesikulärer Marker genutzt (Calakos und Scheller, 1994). Seine Funktion ist bisher allerdings nur wenig verstanden.

Obwohl Deletionsmutanten darauf hinweisen, dass Synaptophysin für die Exozytose scheinbar nicht essentiell ist (Eshkind und Leube, 1995), war nach Injektion von mRNA antisense Nukleotiden und Antikörpern gegen Synaptophysin die Transmitterexozytose eingeschränkt (Alder et al., 1992). Bei gestörter Synaptophysinexpression in neuronalen Zellkulturen ist die Fähigkeit Synapsen zu bilden unter kompetetiven Bedingungen verringert (Tarsa und Goda, 2002). Es konnte auch gezeigt werden, dass Synaptophysinmultimere Ionenkanäle bilden können (Thomas et al., 1988)

Darüber hinaus scheint Synaptophysin in Abhängigkeit von der Reife des Neurons die Verfügbarkeit von Synaptobrevin zu modulieren (Edelmann et al., 1995) und eine Rolle bei der Endozytose und Wiederverwertung der synaptischen Vesikel zu spielen (Daly und Ziff, 2002). Als cholesterolbindendes Protein scheint es für die Biogenese und die hohe Membrankrümmung der synaptischen Vesikel wichtig zu sein (Thiele et al., 2000). Synaptische Vesikel weisen eine überdurchschnittliche Dichte an Cholesterol auf. Cholesterol ist dort aber nicht gleichmäßig verteilt, sondern in so genannten Clustern angehäuft. Die Formation von Proteinen in diesen Zonen ist offenbar für die Funktion des SNARE-Komplexes essentiell (Salaun et al., 2004).

Synaptobrevin ist auf die Formation eines Komplexes beschränkt, es bindet entweder nur Synaptophysin oder im SNARE-Komplex.

Die Interaktion von Synaptophysin mit Synaptobrevin findet sich ausschließlich in adulten Neuronen und konnte weder mit rekombinanten Proteinen noch in embryonalen oder neuroendokrinen Zellen dargestellt werden. Dies beruht offensichtlich auf einer posttranslationalen Modifikation von Synaptophysin, die über einen unbekannten zytosolischen Faktor induziert wird (Becher et al., 1999).

Ziel der vorliegenden Arbeiten war es, die Abhängigkeit der Interaktion von Synaptophysin und Synaptobrevin von verschiedenen Faktoren in mehreren Modellen zu beschreiben und damit die physiologische Funktion des Komplexes zu evaluieren.

Es interessierte dabei speziell, über welche Strukturen die beiden Proteine miteinander agieren und inwiefern sich der Komplex von Stimulation bzw. Inhibition der Exozytose und vom Cholesterolgehalt der Plasmamembran abhängig zeigt.

2 Methoden

2.1 Proteingewinnung, Modellsysteme

Für die Untersuchungen konnten verschiedene Modellsysteme genutzt werden. Zellkulturen hippocampaler Neurone wurden aus Mäuseembryonen gewonnen und bis zu 14 Tage (days in vitro, DIV) kultiviert. Die Behandlung mit verschiedenen Zusätzen erfolgte über den im jeweiligen Experiment angegebenen Zeitraum. Die Reaktion wurde auf Eis gestoppt, die Zellen geerntet und in Puffer mit Proteinaseinhibitoren resuspendiert, homogenisiert, zentrifugiert und extrahiert.

Physiologisch intakte synaptische Endigungen (Synaptosomen) und synaptische Vesikel wurden mittels Homogenisation und mehrerer Zentrifugationsschritte aus dem frischpräparierten Cortex adulter bzw. embryonaler Ratten gewonnen.

Für einige Versuche wurde rekombinantes Synaptobrevin verwendet, das in E. coli vom Stamm M15 exprimiert wurde. Die Konstrukte mit Amino- (N-) bzw. Carboxy- (C-) terminalem 6 Histidin (His)-Tag wurden in den pQE-28a bzw. pQE-30 Vektoren von Quiagen einkloniert und diese in E. coli transfiziert. Stellte sich während der Kultivierung unter photometrischer Messung die stationäre Phase ein, wurde den Kulturen Isopropyl-β-D-Thiogalactopyranosid (IPTG) zugefügt und diese nach weiterem Wachstum sedimentiert. Unter Zusatz von Proteinaseinhibitoren wurde das Pellet in Lyse-Puffer resuspendiert, nach Inkubation mit Lysozym und DNase auf Eis mechanisch lysiert und anschließend mit Triton X-100 extrahiert. Die Isolierung und Aufreinigung der His-getagten Proteine erfolgte über Bindung an Nickel- Nitrilotriessigsäure (Ni-NTA) Säulen und Elution mit Imidazol. Zum Entfernen des für die Versuche störenden Imidazol wurde das Eluat dialysiert.

Die Proteinmenge wurde jeweils mittels BicinChoninic Acid (BCA)-Test ermittelt.

2.2 Toxine und Stimulantien

Um den Synaptophysin/Synaptobrevin-Komplex in seiner Funktion während der Exozytose genauer untersuchen zu können, wurden verschiedene Toxine und Stimulantien als Hilfsmittel benutzt.

Die verwendeten Stimulantien α-Latrotoxin und Ca^{2+}-Ionophore A23187 lösen in vitro eine Exozytose aus. Während α-Latrotoxin über einen bisher noch nicht genau geklärten Mechanismus konzentrationsabhängig und Ca^{2+}-unabhänig zu einer massiven Exozytose der Neurotransmitter führt, wirkt die Ca^{2+}-Ionophore, welche den Transport von Ionen durch die Lipidmembran ermöglicht, schwächer und nur bei Vorhandensein von Calciumionen.

Zur Exozytoseuntersuchung verwendeten wir die clostridialen Neurotoxine Tetanustoxin (TeNt) sowie Botulinumtoxin (BoNT) A, B und D. Die Toxine spalten je nach Typ spezifisch eines der SNARE-Proteine und unterbinden damit die Exozytose vollständig. In einzelnen Versuchen wurde nur die leichte Kette des TeNt verwendet. Das in den Versuchen verwende-

te Botulinumtoxin A wirkt spezifisch auf SNAP25, während Tetanustoxin sowie Botulinumtoxin B und D die Spaltung von Synaptobrevin katalysieren.

Rekombinant hergestellte Konstrukte, bestehend aus Botulinumtoxin vom Serotyp D (BoNT/D) und verschiedenen, an das N-terminale Ende gekoppelten „Cargo“-Proteinen (unter anderen Green Fluorescent Protein, GFP) wurden in Kontinuität (als single chain, scBoNT) exprimiert. In vivo wird das Toxin zwischen der leichten und der schweren Kette proteolytisch gespalten („nicked“ BoNT), so dass diese nur noch durch eine Disulfidbrücke und nicht-kovalente Bindungen zusammenhängen. Die Spaltung konnte bei allen rekombinanten Toxinen künstlich herbeigeführt werden, ohne dabei die fusionierten Proteine freizusetzen.

Der Vergleich von BoNT/D und GFP-BoNT/D in genickter und ungenickter Form erfolgte in hippocampalen Neuronen in verschiedenen Konzentrationen nach 1 bis 6 DIV. Zur Blockade des natürlichen Aufnahmeweges wurde Bafilomycin A1 verwendet.

2.3 Cholesterol

Die Verminderung der Cholesterolkonzentration wurde unter Verwendung von Lovastatin in Neuronen sowie mit Filipin und β-Methylcyclodextrin (β-MCD) bei synaptischen Vesikeln erreicht. Alternativ wurden die Konzentration durch Zugabe von Cholesterol zu den Zellkulturen am 1. DIV (days in vitro) erhöht und die Neurone bis zum DIV 5 bzw. 11 kultiviert.

2.4 Immunpräzipitation, Crosslinking, Bead-Versuch, Western Blot, Quantifizierung

Die Immunpräzipitation erfolgte durch Inkubation von Triton X-100 Extrakt mit monoklonalem Antikörper und anschließender Separierung über G-Sepharose. Das über Zentrifugation gewonnene Pellet wurde gewaschen. Danach wurden Pellet und Überstand in Probenpuffer resuspendiert und getrennt ausgewertet.

Zum Crosslinking der synaptischen Proteine verwendeten wir Disuccinimidylsuberat (DSS).

Rekombinantes Synaptobrevin mit N- bzw. C-terminalem His-Tag wurde an Ni-NTA Säulen fixiert und wahlweise mit der isolierten leichten Kette des Tetanustoxins (TeNt-LC) gespalten. Nicht gebundene Proteine und Fragmente wurden durch Waschen entfernt. Die Säulen wurden daraufhin mit dem Extrakt vesikulärer Membranen (lösliche Proteine) inkubiert und nach mehreren Waschschritten eluiert. Die gebundenen Proteine wurden über das Westernblot Verfahren dargestellt.

Die molekulargewichtabhängige Auftrennung der Proteine erfolgte über SodiumDodecylSulfat-PolyAcrylamidGelElektrophorese (SDS-Page) mit Trenngelen zwischen 10% und 15%. Die Proteine wurden im SemiDry Blot-Verfahren auf Nitrocellulose transferiert und über Immundetektion mit Enhanced Chemiluminiscence (ECL) ausgewertet. ECL-Filme wurden eingescannt und die Proteinbanden mit Scan Pack 3.0 densitometrisch quantifiziert. Die statistische Auswertung erfolgte über den Stundent’s T-Test.

2.5 Antikörper

Zur Analyse der Immunoblots und für die Immunpräzipitation wurden monoklonale Antikörper gegen Synaptobrevin 2 (Klon 69.1), Synaptophysin (Klon 7.2), Syntaxin1a/b (Klon HPC-1), SNAP25 (Klon 71.1) sowie durch BoNT/A gespaltenes SNAP25 (Klon p1-16) verwendet. Polyklonale Antikörper wurden zur Darstellung von Synaptophysin (Klon G96) und Synaptobrevin (Klon 106.5) genutzt. Als sekundäre Antikörper kamen mit Meerrettich-Peroxidase gekoppelte Pferd anti-Maus und Ziege anti-Hase Seren zum Einsatz.

Synaptobrevin 2/VAMP 2 ist die am meisten vorkommende der 3 Isoformen, und wird im Folgenden nur noch als Synaptobrevin bezeichnet.

3 Ergebnisse

3.1 Verhalten vom Synaptophysin/Synaptobrevin-Komplex nach Stimulation

Aus eigenen Untersuchungen an hippocampalen Neuronen ergab sich, dass deren kurzzeitige Stimulation mit α-Latrotoxin zu einer zeit- und konzentrationsabhängigen Abnahme der Interaktion zwischen Synaptophysin und Synaptobrevin führte. Die Interaktion von SNAP25 mit Synaptobrevin bzw. Syntaxin nahm hierbei allerdings nur geringgradig zu. Auch die Stimulation mit Ca^{2+}-Ionophore bewirkte eine Dissoziation des Komplexes, die jedoch unter Entzug von Calcium ausbleibt. Wurde vor der Stimulation mit α-Latrotoxin und Ca^{2+}-Ionophore die Vesikelexozytose durch Behandlung der Neurone mit Botulinumtoxin A (BoNT/A) blockiert, kam es ebenfalls zu einer Reduktion des Synaptophysin/Synaptobrevin-Komplexes. Die alleinige Behandlung mit BoNT/A hatte jedoch auch nach mehrtägiger Inkubation keinen Einfluss auf die Synaptophysin/Synaptobrevin-Komplexbildung. Diese Ergebnisse zeigen erstmalig, dass die durch Exozytosestimuli ausgelöste Dissoziation des Komplexes der finalen Membranfusion voraus geht und von ihr unabhängig ist.

Der Funktionsnachweis von BoNT/A ergab sich einerseits aus dem sichtbaren Shift der SNAP25-Bande in der Elektrophorese und gelang andererseits durch die Darstellung von gespaltenem SNAP25 mit Hilfe des speziellen Antikörpers p1-16. Dieser monoklonale Antikörper erkennt eine Proteinsequenz am Ende von SNAP25, die durch die Spaltung freigelegt wird. Darüber hinaus konnte in eigenen Experimenten mit diesem Antikörper demonstriert werden, dass gespaltenes SNAP25 über die Immunpräzipitation mit Antikörpern gegen Synaptobrevin extrahiert werden kann.

Unsere Arbeitsgruppe konnte in Versuchen mit Synaptosomen zwar die Labilität des Synaptophysin/Synaptobrevin-Komplexes durch die reversible Dissoziation nach Behandlung mit hoch konzentrierter NaCl-Lösung bzw. mit β-Mercaptoethanol zeigen, konnte jedoch keine direkte Wirkung von ionischem Calcium auf den Komplex feststellen.

3.2 Einfluss von Cholesterol auf den Komplex

Ein weiteres Ergebnis der durchgeführten Versuche ist der Nachweis, dass die Interaktion von Synaptophysin und Synaptobrevin vom Cholesterolgehalt der Vesikelmembran abhängt. Nach Cholesteroldepletion sowohl mit Filipin bzw. β-MCD in Synaptosomen als auch unter Lovastatinbehandlung in neuronalen Zellkulturen konnte über Immunpräzipitation eine eindeutige Abnahme des Synaptophysin/Synaptobrevin-Komplexes festgestellt werden. Die Interaktion von Synaptobrevin und SNAP25 blieb in diesen Ansätzen nahezu unverändert. Wurde dagegen Cholesterol den im Wachstum befindlichen hippocampalen Neuronen zugesetzt, steigerte dies nach mehrtägiger Behandlung die Interaktion von Synaptophysin und Synaptobrevin im Vergleich zu unbehandelten Zellkulturen signifikant.

Die Cholesterolabhängigkeit der Komplexbildung konnte von unserer Arbeitsgruppe auch im Tiermodell bestätigt werden. Ebenso konnte die unterschiedliche Extrahierbarkeit von Synaptophysin in Abhängigkeit vom Cholesterolgehalt der Membran gezeigt werden. Eine Analyse des Cholesterolgehaltes der Membranen von embryonischen synaptischen Vesikeln ergab einen 3-fach geringeren Cholesterolanteil im Vergleich zu adulten Vesikeln.

Zusammenfassend belegen diese Experimente, die wichtige Rolle, die Cholesterol für die Ausbildung des Synaptophysin/Synaptobrevin Komplexes und die Reifung der synaptischen Vesikel spielt.

3.3 Interaktion von Synaptobrevin mit Synaptophysin

Synaptobrevin war nach mehrtägiger Inkubation einer Zellkultur hippocampaler Neurone mit Tetanustoxin (TeNt) erwartungsgemäß nicht mehr detektierbar. Sowohl die Expression von Synaptophysin als auch die Komplexbildung von SNAP25 und Syntaxin blieben hierbei jedoch unverändert. Während beschrieben wurde, dass Synaptobrevin im SNARE-Komplex vor der enzymatischen Spaltung durch Tetanustoxin geschützt ist (Hayashi et al., 1994), konnte unsere Arbeitsgruppe nach Crosslinking und Inkubation von Synaptosomen mit TeNt zeigen, dass Synaptobrevin im Komplex mit Synaptophysin weiterhin durch TeNt angreifbar ist und in diesem im Vergleich zum Monomer sogar bevorzugt gespalten wird.

Dieser Aspekt erwies sich als geeignet um zu eruieren, ob nach der Spaltung eines der Synaptobrevinfragmente in Bindung an Synaptophysin nachweisbar ist. Um dies zu analysieren wurden Synaptosomen nach dem Crosslinking mit TeNt oder BoNT/D behandelt. Dabei zeigte sich in der Elektrophorese ein neuer Komplex aus Synaptophysin und einem der beiden neu entstandenen Synaptobrevinfragmente. Über die unterschiedliche Laufzeit und Darstellbarkeit mit verschiedenen Antikörpern konnte das C-terminale Fragment als das bindende identifiziert werden.

Unter Verwendung von rekombinant hergestellten Synaptobrevin-Konstrukten mit N- bzw. C-terminalem His-Tag konnte die Interaktion von Synaptobrevin mit den präsynaptischen Proteinen auch direkt überprüft werden. Beide Konstrukte hatten unveränderte Bindungseigenschaften zu den SNARE-Proteinen SNAP25 und Syntaxin als auch zu Synaptophysin. Über

den N-terminalen His-Tag fixiertes und mit Hilfe von TeNT gespaltenes Synaptobrevin hat nach Inkubation mit Extrakt aus adulten Synaptosomen SNAP25 und Syntaxin unverändert gebunden. Die Bindung von Synaptophysin nahm durch die Spaltung hingegen ab. Demgegenüber wies das Konstrukt mit dem C-terminalen Tag nach der Spaltung eine verminderte Bindung der SNARE-Proteine auf. Die Bindung von Synaptophysin war im Vergleich zum nicht gespaltenen Synaptobrevin unvermindert. Damit konnte die Bindung von Synaptophysin an Synaptobrevin über dessen C-Terminus bewiesen werden.

3.4 Botulinumtoxin als Werkzeug

Andere Experimente betrafen die Verwendung von Botulinumtoxin als Werkzeug für Untersuchungen der Vesikelexozytose. Als Ergebnis dieser Experimente ergab sich, dass ein an die leichte Kette von BoNT/D gehängtes Protein spezifisch in Neurone transloziert wird und dort seine enzymatische Aktivität entfalten kann. Die Fähigkeit der Konstrukte ins Zytosol zu translozieren hing dabei weniger von der Größe der angehängten Proteine, als vielmehr von der Stabilität ihrer Konformation ab.

BoNT/D, an dessen leichte Kette das Protein GFP angehängt wurde, hat in eigenen Versuchen an hippocampalen Neuronen im Vergleich zu nativem BoNT/D eine geringere Toxizität. Wie sich in der zeitabhängigen Vergiftungsreihe zeigt, beruht dies eher auf einer verlangsamten Translokation ins Zytosol als auf einer gestörten Funktion. Weiterhin konnte man beim Vergleich von rekombinantem scBoNT/D mit nativem, genicktem BoNT/D erkennen, dass allein die enzymatische Trennung der leichten von der schweren Kette (nicking) eine Zunahme der Toxizität um ein Mehrfaches bewirkt.

BoNT kann nur dann als spezifischer Transporter genutzt werden, wenn durch die Kopplung mit dem Cargo-Protein kein alternativer Aufnahmeweg möglich ist. In eigenen Experimenten konnte gezeigt werden, dass über die Blockade der vesikulären H^+-ATPase durch Bafilomycin A1 sowohl die Wirkung von BoNT/D als auch GFP-BoNT/D eindeutig verringert wurde. Dies gibt Anhalt dafür, dass die Verlagerung der katalytischen Domäne ins Zytosol durch saure Kompartimente erfolgt und dieser Pfad bei den Fusionsproteinen nicht verändert ist.

4 Diskussion

Die vorgestellten Ergebnisse zeigen, dass der Synaptophysin/Synaptobrevin-Komplex in Abhängigkeit von der synaptischen Aktivität dynamisch reguliert wird. Verschiedenartige Kurzzeitstimulationen lösen über einen bisher nicht identifizierten Ca^{2+}-abhängigen Schritt die Dissoziation des Synaptophysin/ Synaptobrevin-Komplexes aus. Die Veränderung der Interaktion der beiden Proteine durch den Exozytosestimulus ist auch durch BoNT/A nicht blockierbar, ist also unabhängig von der finalen Membranfusion. Dies ist mit der These vereinbar, dass der Komplex einen Reservepool zur Bereitstellung von Synaptobrevin für den SNARE-Komplex darstellt und in Vorbereitung auf die Exozytose von bislang unbekannten Faktoren moduliert wird. Allerdings weist die fehlende Interaktion von Synaptophysin und

Synaptobrevin in embryonalen und neuroendokrinen Zellen (Becher et al., 1999) darauf hin, dass Synaptophysin noch andere Aufgaben neben der Interaktion mit Synaptobrevin hat.

Während die direkte Zugabe von Ca^{2+} in Versuchen mit synaptosomalen Extrakten zu einer Abnahme der Komplexmenge führte (Daly und Ziff, 2002; Prekeris und Terrian, 1997), hatte es in unseren Experimenten mit Extrakt aus synaptischen Vesikeln keinen Effekt. Dies lässt vermuten, dass das in den Synaptosomen enthaltene Zytosol einen zusätzlichen Faktor enthält, der die calciumabhängige Dissoziation vermittelt.

Eine von der Exozytose unabhängige Dissoziation des Komplexes nach α-Latrotoxin Stimulation konnte auch mittels Fluorescence Resonance Energy Transfer Analyse gezeigt werden (Pennuto et al., 2002). Dagegen fand sich bei Untersuchungen mit membranpenetrierendem Crosslinkingverfahren an Synaptosomen ein Anstieg des Synaptophysin/Synaptobrevin-Komplex nach Stimulation (Khvotchev und Sudhof, 2004). Diese Arbeitsgruppe wies weiterhin nach, dass die Komplexbildung von der Intaktheit der Membran abhängt und damit die Extraktion mit von uns genutzten Detergenzien die Bindungseigenschaften der Proteine verändern könnte. Andererseits fand in diesen Versuchen das Crosslinking während der Stimulation statt. Im theoretischen Falle eines hohen turn over des Komplexes mit kombinierter Assoziation und Dissoziation, würde durch das Crosslinking die Trennung verhindert und eine Nettodissoziation wäre damit maskiert. In diesem Sinne könnte man vermuten, dass Synaptophysin vor der Exozytose von Synaptobrevin abdissoziert und während oder nach der Endozytose wieder an das vesikuläre SNARE-Protein zu binden um die gezielte schnelle Wiederaufnahme und Sortierung von Synaptobrevin in die Vesikel zu ermöglichen. Dafür spricht auch, dass Synaptophysin in der Lage ist die Wiederaufnahme von Synaptobrevin 2 in synaptische Vesikel zu bewirken (Pennuto et al., 2003).

Die Beobachtung, dass es nach Langzeitstimulation zu einer Steigerung des Synaptophysin/Synaptobrevin-Komplexes kommt (Hinz et al., 2001) kann auch damit erklärt werden, dass der Komplex als Reservepool fungiert und unter diesen Bedingungen hochreguliert wird. Da Synaptophysin auch eine Rolle bei der schnellen Endozytose spielt (Daly und Ziff, 2002), ist die Zuteilung von Synaptobrevin zu synaptischen Vesikeln durch diese Interaktion denkbar.

Der Entzug von Cholesterol führt zur Inhibition der regulierten Exozytose (Kato et al., 2003). Wir konnten zeigen, dass Cholesterol in synaptischen Vesikeln und Zellkulturen auch einen Einfluss auf die Interaktion von Synaptophysin und Synaptobrevin hat. Die Verringerung des Cholesterolgehaltes der Membranen vermindert die Komplexbildung, bei Substitution mit Cholesterol ist sie erhöht. Da der Cholesterolgehalt der Membranen während der neuronalen Entwicklung steigt, korreliert die Komplexmenge dementsprechend mit der synaptischen Reifung.

Synaptophysin ist ein Cholesterol-bindendes Protein (Thiele et al., 2000) und ist im Gegensatz zu anderen synaptischen Proteinen nach Cholesteroldepletion schlechter löslich. Es könnte daher an der Bildung von Membranclustern beteiligt sein, die Anordnung und Interaktion von vesikulären Proteinen fördern.

Die Abhängigkeit der Komplexbildung vom Cholesterolgehalt der Membran gibt außerdem Anhalt für eine Interaktion der beiden Vesikelproteine im Bereich der Transmembranregion. Alternativ wäre auch eine notwendige Annäherung über Membrancluster denkbar. Um die für die Interaktion benötigte Domäne zu spezifizieren, führten wir verschiedene Bindungsstudien mit rekombinantem kompletten als auch mit gespaltenem Synaptobrevin durch. Über zwei verschiedene Ansätze konnten wir zeigen, dass hauptsächlich der C-Terminus des Synaptobrevin für die Bindung zwischen den beiden Vesikelproteinen sorgt.

Der Nachweis, dass der Komplex unter hoher Salzkonzentration und reduzierende Bedingungen dissoziiert, spricht für eine Interaktion der Proteine in der zytosolischen Domäne. In Kombination mit den Bindungsstudien lasst dies vermuten, dass es sich um den Bereich nahe der Transmembranregion handelt.

Nicht im Einklang mit unseren Ergebnissen scheint die Tatsache, dass Synaptobrevin, welches über den N-Terminus am SNARE-Komplex partizipiert, nicht in der Lage ist Synaptophysin zu binden. Weiterhin bewirkt die Einführung von N-terminalen Synaptobrevinfragmenten (AS 1-32) die Aufhebung der Bindung an Synaptophysin (Washbourne et al., 1995) und in Immunfluoreszenzversuchen erwies sich der zytoplasmatische Teil von Synaptobrevin 2 als essentiell für die Synaptophysin-abhängige Sortierung zu synaptischen Vesikeln (Pennuto et al., 2003). Jedoch verwenden diese Versuche kompetetive Bedingungen und konnten keine direkte Bindung nachweisen.

Die Untersuchung der Wirkungsweise von Botulinumtoxin verhilft zu neuen Techniken, um Proteine gezielt in Zellen zu transportieren. Unsere Darstellung der veränderten Toxizität in Abhängigkeit vom nicking oder angehängten Proteinen ist hilfreich für weitere Versuche, in denen eine effektive Wirkung der Toxine angestrebt wird bzw. ermöglicht eine effektive Translokation ohne toxische Nebeneffekte.

Der Nachweis, dass die Kopplung von Proteinen an proteolytisch inaktive clostridiale Toxine ein geeignetes Mittel für den Transport in das Zytosol neuronaler Zellen darstellt, ist aus mehreren Gründen von Vorteil. Einerseits kann man dieses Modul als spezifischen Transporter für noch zu entwickelnde Pharmaka verwenden, andererseits erhält man damit ein nützliches Werkzeug für die Untersuchung der Prozesse an der Synapse. Mit Hilfe dieses Transporters wäre es z.B. möglich den Faktor, der die Bindung zwischen Synaptophysin und Synaptobrevin in adulten Neuronen vermittelt, sollte er sich denn als Protein herausstellen, ins Zytosol anderer Zellen zu translozieren und diese daraufhin auf veränderte Eigenschaften zu untersuchen.

In eigenen wie auch in den zitierten Studien zeigte sich eine veränderte Interaktion zwischen Synaptophysin und Synaptobrevin in Abhängigkeit von Stimulation, neuronaler Reifung, Bestandteilen der Zellmembran oder des Zytosols und anderen Faktoren. Damit zeugen die Arbeiten vom modulierenden Charakter von Synaptophysin. Was diese Modulation bewirkt ist weiterhin ungewiss. Beeinflusst der Synaptophysin/Synaptobrevin-Komplex die Ausbildung des SNARE-Komplexes positiv oder negativ? Fungiert er als Reservepool für die effek-

tive Bereitstellung von Synaptobrevin oder verhindert er, dass Synaptobrevin nach ATP-verbrauchender Auflösung des SNARE-Komplexes wieder in diesen eintritt?

In den vorliegenden Arbeiten finden sich überwiegend Hinweise auf den positiv modulatorischen Effekt von Synaptophysin. Die Dissoziation des Komplexes vor der Exozytose ermöglicht Synaptobrevin leichter in den für die Exozytose essentiellen SNARE-Komplex einzutreten. Auch das Ergebnis, dass die Interaktion von Synaptophysin mit dem C-terminalen Part erfolgt, stützt die Theorie der Bereitstellung von Synaptobrevin für den SNARE-Komplex. Die Bindungsdomäne am N-terminalen Ende des Proteins, das für die Interaktion mit dem Membrankomplex verantwortlich ist, wäre damit frei verfügbar.

Mit Hilfe unserer Arbeiten konnte der Komplex der Vesikelproteine Synaptophysin und Synaptobrevin genauer charakterisiert werden, der genaue Nachweis seiner funktionellen Bedeutung steht noch aus.

Anmerkung: In der Zusammenfassung wurden die Ergebnisse aus den Veröffentlichungen, die nicht direkt aus meinen Versuchen hervorgingen verkürzt erläutert. Eigene Ergebnisse wurden detailliert dargestellt.

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5 Veröffentlichungen

Diese Publikationsdissertation basiert auf folgenden Veröffentlichungen:

“The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content.”

Mitter D, Reisinger C, Hinz B, Hollmann S, Yelamanchili SV, Treiber-Held S, Ohm TG, Herrmann A, Ahnert-Hilger G.

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The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content

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Abstract

Synaptophysin interacts with synaptobrevin in membranes of adult small synaptic vesicles. The synaptophysin/synaptobrevin complex promotes synaptobrevin to built up functional SNARE complexes thereby modulating synaptic efficiency. Synaptophysin in addition is a cholesterol-binding protein. Depleting the membranous cholesterol content by filipin or β-methylcyclodextrin (β-MCD) decreased the solubility of synaptophysin in Triton X-100 with less effects on synaptobrevin. In small synaptic vesicles from rat brain the synaptophysin/synaptobrevin complex was diminished upon β-MCD treatment as revealed by chemical cross-linking. Mice with a genetic mutation in the Niemann–Pick C1 gene developing a defect in cholesterol sorting showed significantly reduced amounts of the synaptophysin/synaptobrevin complex compared to their homo- or heterozygous littermates. Finally when using primary cultures of mouse hippocampus the synaptophysin/synaptobrevin complex was down-regulated after depleting the endogenous cholesterol content by the HMG-CoA-reductase inhibitor lovastatin. Alternatively, treatment with cholesterol up-regulated the synaptophysin/synaptobrevin interaction in these cultures. These data indicate that the synaptophysin/synaptobrevin interaction critically depends on a high cholesterol content in the membrane of synaptic vesicles. Variations in the availability of cholesterol may promote or impair synaptic efficiency by interfering with this complex.

Keywords: cholesterol, small synaptic vesicles, synapse maturation, synaptophysin, synaptobrevin.

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Regulated release of neurotransmitters from synaptic vesicles is essential for neuronal communication. A key event involved in the highly controlled interaction between the vesicular membrane and the plasma membrane is the formation of the ternary SNARE complex that is built up by the vesicular membrane protein synaptobrevin and the plasma membrane associated proteins SNAP25 and syntaxin (Sutton *et al*. 1998; Jahn and Südhof 1999; Lin and Scheller 2000). Besides being a member of the SNARE complex, synaptobrevin also interacts with the vesicular membrane protein, synaptophysin. The two complexes are mutually exclusive. Thus synaptophysin might control the availability of synaptobrevin to enter the SNARE complex (Edelmann *et al*. 1995). Synaptophysin probably supports the interaction of synaptobrevin with its SNARE partners as it enhances exocytosis at the neuromuscular junction (Alder *et al*. 1992, 1995). Indeed, the synaptophysin/synaptobrevin complex up-regulates during neuronal development (Becher *et al*. 1999a) and is increased in adult brain during long-lasting, generally enhanced synaptic activity (Hinz *et al*. 2001). In addition, wild-type synapses expressing synaptophysin are better donors of pre-synaptic terminals when compared with synaptophysin-deficient synapses (Tarsa and Goda 2002). These data support the idea that synaptophysin is not absolutely required but may represent a positive modulator of exocytosis.

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Abbreviations used: BCA, bicinchoninic acid; β-MCD, β-methylcyclodextrin; CHO, Chinese hamster ovary; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; DRM, detergent-resistant membrane; DSS, disuccinimidyl suberate; E, extract; ECL, enhanced chemiluminescence; IP, immunoprecipitate; NE, non-extract; NPC1, Niemann–Pick C1 protein; NSF, *N*-ethylmaleimide sensitive factor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor complex; SSV, small synaptic vessicle.

Synaptophysin has been reported to bind cholesterol selectively and thus probably represents a key protein for the biogenesis of synaptic vesicles (Thiele *et al.* 2000). Whether the cholesterol binding might be also crucial for other functions of synaptophysin is still an open issue. Studies from plasma membrane proteins revealed that transmembrane and membrane attached proteins, which are often palmitoylated, form functional complexes by aggregating in specialized areas of the membrane called micro domains or lipid rafts. These rafts are characterized by high cholesterol and sphingolipid content and an insolubility in detergents like Triton X-100. Whether raft-like membrane domains also apply to synaptic vesicles is not known so far.

Assuming that for fusion to occur almost all vesicular synaptobrevin molecules have to be positioned in such a way that they can interact with the plasma membrane proteins syntaxin and SNAP25, this configuration might require a specific lateral organization of the synaptic vesicle membrane. The ability of synaptophysin to bind cholesterol might be crucial for such an alignment. The present study used various *in vitro* and *in vivo* approaches to analyse the effects of an overall decrease or increase in cholesterol content and to explore whether synaptophysin/synaptobrevin interaction alters with such changes.

Materials and methods

Antibodies

The following antibodies were kindly supplied by R. Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany); mouse monoclonal antibodies against synaptobrevin II (clone 69.1, Edelmann *et al.* 1995) and synaptophysin (clone 7.2, Jahn *et al.* 1985). Monoclonal antibodies against synaptogyrin and syntaxin1 A/B (clone HPC-1) were purchased from Synaptic Systems (Göttingen, Germany) and anti-SNAP25 from Sternberger Monoclonals (Baltimore, MD, USA).

Secondary antibodies for western blot detection, horse anti-mouse and goat anti-rabbit conjugated either with horseradish peroxidase or alkaline phosphatase were purchased from Vector Laboratories (Burlingame, CA, USA).

Other chemicals

β-Methylcyclodextrin (β-MCD), cholesterol, lovastatin (mevostatin) and filipin were purchased from Sigma (St Louis, MO, USA). Cholesterol was dissolved in ethanol at a concentration of 30 mg/mL and used at final concentrations between 10 and 20 μg/mL.

Synaptosomes and synaptic vesicles

Isolated nerve terminals (crude synaptosomes) were prepared at 4°C from adult rat or mouse whole brains in the presence of protease inhibitors (Edelmann *et al.* 1995; Becher *et al.* 1999a). Crude synaptic vesicles (LP2 fraction) were prepared from adult and embryonic brains following the procedure described (Huttner *et al.* 1983). Crude synaptosomes or synaptic vesicles (1–2 mg of protein) were incubated with β-MCD or filipin to reduce the membrane cholesterol before extracting them in 1 mL extraction buffer containing: KCl 140 mM, EDTA 2 mM, HEPES–KOH 20 mM, pH 7.3, and 1% (v/v) Triton X-100. Extraction was performed for 1 h at 4°C under rotation, followed by centrifugation for 3 min at 700 *g*. Then, 2.5 μL of ascites fluid (corresponding to about 7.5 μg of IgG) of the monoclonal antibodies against either synaptobrevin or synaptophysin were added to 200 μL of extract. Incubation was carried out for 16–18 h at 4°C. Immunoprecipitates were separated by addition of 25 μL of G-Sepharose Fast Flow beads (Pharmacia Biotech, Piscataway, NJ, USA). The beads were then pelleted for 1 min at 200 *g and* washed three times in extraction buffer. Finally, the beads were resuspended in sample buffer, boiled for 5 min and then analysed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blotting. The supernatant from the immunoprecipitation was analysed in parallel. For cross-linking experiments synaptic vesicles were resuspended in Krebs–Ringer buffer containing: NaCl 140 mM, $NaHCO_3$ 5 mM, $MgCl_2$ 1 mM, Na_2HPO_4 1.2 mM, glucose 10 mM, HEPES–NaOH 20 mM, pH 7.4. Synaptic vesicles (1.5 mg/mL protein) were incubated with the chemical cross-linker disuccinimidyl suberate (DSS) dissolved in dimethyl sulfoxide, to yield a final concentration of 0.5 mM. After incubation at room temperature for 45 min under rotation, the reaction was quenched by incubating for 30 min with Tris–HCL pH 7.4 (final concentration of 100 mM), membranes were subsequently pelleted at 350 000 *g* for 30 min and analysed by SDS–PAGE and immunoblotting under non-denaturating conditions.

Enhanced chemiluminescence (ECL)-processed films were scanned by video imaging and protein bands were densitometrically quantified using SCAN PACK 3.0 program (Whatman Biometra GmbH, Göttingen, Germany). In experiments performed with cross-linking technique, ratio between the arbitrary units of synaptophysin and synaptophysin/synaptobrevin complex was calculated using the synaptophysin antibody for detection. Alternatively, when detecting with the antibody against synaptobrevin, the ratio between synaptobrevin and the synaptophysin/synaptobrevin complex was calculated. Quantification after immunoprecipitation was performed as given (Hinz *et al.* 2001) along with statistical analysis using the paired Student's *t*-test type 3.

Cell culture

The cell lines Chinese hamster ovary (CHO) p38 or PC12 were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum or DMEM with 10% horse serum and 5% fetal calf serum, respectively, and kept in an atmosphere of 10% CO_2. Primary cultures from embryonic (embryonic day 17) mouse hippocampal neurones were prepared and cultivated up to 12 days *in vitro* (DIV; Grosse *et al.* 2000).

Membrane preparations

Cells were suspended in phosphate-buffered saline (PBS) and lysed by adding 9 parts of water supplemented with protease inhibitors [pepstatin/leupeptin and phenylmethylsulphonyl fluoride (PMSF)]. This suspension was first homogenized (9 strokes at 900 rpm) and then subsequently passed 9 times through a 23-G and 6 times through a 27-G needle followed by centrifugation at 12 000 *g* to discard the nuclear debris. The supernatant was centrifuged for 30 min at 220 000 *g* and the pellet was resuspended in PBS and

distributed to individual tubes supplemented by the substances to be tested. Incubation was performed at 37°C for 20 min, stopped by centrifugation at 220 000 *g* for 30 min and the membranes were finally subjected to the extraction procedure (see above). Protein content was measured using the BCA (bicinchoninic acid) test (Sigma). Equal amounts of synaptosomal or vesicular proteins were subjected to the various incubations followed by either the cross-linking or the extraction/immunoprecipitation procedure.

Determination of cholesterol content and cholesterol depletion

After lipid extraction according to Bligh and Dyer (1959), cholesterol was quantified by a colourimetric method based on the conversion of cholesterol to cholestenone by cholesterol oxidase (kit from Boehringer Mannheim/R-Biopharm, Darmstadt, Germany).

Cholesterol depletion was achieved by incubating membranes, synaptosomes or synaptic vesicles with either filipin or β-MCD before they were subjected to extraction followed by immunoprecipitation or chemical cross-linking.

Niemann–Pick C1 mice

Breeding-pairs of BALB/cNctr-npc1N heterozygous mice for Niemann–Pick C (NPC; $^{+vpc}/_{-vpc}$) were purchased from Jackson Laboratories (Bar Harbour, MA, USA). Genotypes were determined by PCR which identified the homozygous NPC1 mutants by a single band at 1209 bp, while the heterozygous NPC1 mice showed two bands at 1209 bp and 1048 bp (Loftus *et al.* 1997). Homozygous, heterozygous and wild-type animals were killed at 9–10 weeks of age when the homozygous mutant animals showed fully developed clinical NPC phenotype as described (Higashi *et al.* 1991).

Results

As a first approach, we analysed the solubility of synaptophysin after treatments known to reduce membrane cholesterol using the synaptophysin expressing CHOp38 (Jahn *et al.* 1985) and the neuroendocrine cell line PC12. Incubation of membrane preparations from CHOp38 or PC12 cells with filipin concentrations ranging between 4 and 100 µg/mL followed by Triton X-100 extraction dramatically decreased the solubility of synaptophysin. Extraction of synaptophysin from control membranes was nearly complete as all synaptophysin was found in the extract (E) and only traces in the remaining non-extract (NE), after incubation with filipin most of the synaptophysin was found in the NE-fraction. In three different membrane preparations the amount of synaptophysin in extracts from filipin-treated membranes decreased by 50% compared to control membranes [synaptophysin in extract 84.3 ± 8.6% for control and 42 ± 9.2% for filipin (100 µg/mL), $n = 3$, $p = 0.01$]. Similar data were obtained when using membranes from PC12 cells where other synaptic proteins like synaptobrevin, syntaxin and SNAP25 could be analysed for comparison. In contrast to synaptophysin their solubility changed less upon filipin treatment (Fig. 1a). Treatment with β-MCD that selectively binds cholesterol, thereby extracting it from membranes, also changed the solubility of synaptophysin in Triton X-100 extracts obtained

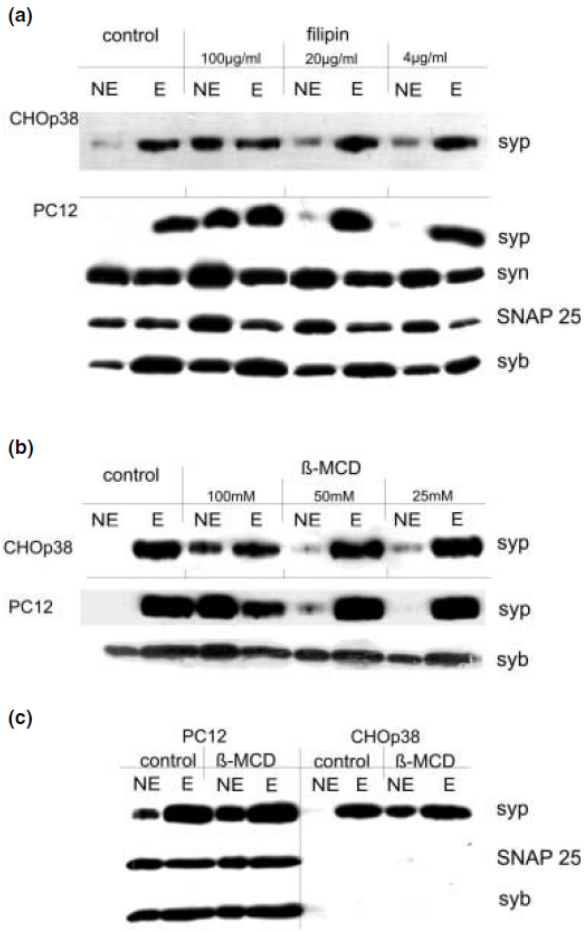

Fig. 1 Decreased solubility of synaptophysin after cholesterol depletion by filipin (a) or β-MCD (b and c). Membranes from CHOp38 or PC12 cells were incubated at 37°C for 60 min with the indicated amounts of filipin or β-MCD before extraction. Corresponding amounts of extraxt (E) and non-extract (NE) were analysed by immunoreplica using antibodies against the proteins indicated at the right side. Syp (synaptophysin); syn (syntaxin); syb (synaptobrevin). In (c) a direct comparison between the two membrane preparations is shown. The following amounts of protein were detected in the extract: (1) membranes from PC12 cells (given in percentage values of the amounts in E plus NE); synaptophysin: control 61% and β-MCD 44%; SNAP25 control 54% and β-MCD 59%; synaptobrevin control 61% and β-MCD 69%; (2) membranes from CHOp38 cells (given in percentage values of the amounts in E plus NE); synaptophysin: control 93% and β-MCD 50%.

from CHOp38 and PC12 membranes, with little change in the solubility of synaptobrevin (Fig. 1b). A direct comparison of the extraction behaviour after β-MCD-treatment of PC12 and CHOp38 membranes is given in Fig. 1(c). In both membrane preparations the extractability of synaptophysin is affected by cholesterol depletion. The decreased solubility of

synaptophysin after cholesterol depletion is slightly affected by the presence of the SNARE proteins synaptobrevin or SNAP25 only expressed in PC12 cells. As observed after filipin-treatment, synaptobrevin and SNAP25 solubility did not alter after the treatment with β-MCD.

Next we analysed the consequences of cholesterol depletion on crude synaptic vesicle preparation (LP2) from adult rat brain. We observed that β-MCD (30 mM) reduced the solubility of synaptophysin and slightly increased the amount of synaptobrevin, synaptogyrin and SNAP25 in the NE fraction (Fig. 2a). However, only synaptophysin was significantly less extractable after treatment with β-MCD (Table 1a). In synaptic vesicles from adult brain the synaptophysin/synaptobrevin interaction was analysed by immunoprecipation using anti-synaptopbrevin antibody. A clear synaptophysin signal could be detected in the synaptobrevin immunoprecipitate (IP) of control membranes but was not to be seen when immunoprecipitating β-MCD-treated membranes (Fig. 2a). Similar results were obtained when using filipin-treated small synaptic vesicle (SSV) membranes (Fig. 2b). The interaction between synaptobrevin and SNAP25 appeared to be almost unaffected. Assuming that the reduced solubility of synaptophysin might lead to the reduction of the synaptophysin/synaptobrevin complex, the interaction of the two proteins was analysed by chemical cross-linking using DSS. The synaptophysin/synaptobrevin complex detected by both antibodies at 56 kDa was reduced (Fig. 2a, right). As little as 2.5 mM β-MCD was sufficient to affect the synaptophysin/synaptobrevin complex in the crude SSV preparation from rat brain (Fig. 3). In a variety of synaptic vesicle preparations the synaptophysin/synaptobrevin complex detected by either antibody was reduced by almost 50% after β-MCD-treatment (Table 1b).

We then investigated SSV from mice with a genetic defect in the murine variant of the NPC1 protein by the cross-linking approach to see whether an abundant availability of cholesterol is crucial for the synaptophysin/synaptobrevin interaction. The phenotype of these mice is characterized by a general delayed development and a reduced motor control. The molecular basis is an impaired function of the NPC1 protein that mainly occurs in astrocytes which are responsible for cholesterol transport out of the late endosomal/lysosomal compartment (Patel *et al.* 1999). As a consequence, cholesterol is trapped in the lysosomal compartments from where it cannot be recruited for neuronal membranes, thus leading to neuronal degeneration (Ong *et al.* 2001; Schroeder *et al.* 2001). No significant difference in the synaptophysin/synaptobrevin complex was observed when comparing healthy homozygous (wt) with heterozygous littermates (het; Fig. 4a, left panel). By contrast, a reduction of the synaptophysin/synaptobrevin interaction was observed when analysing SSV from homozygous mutant litter mates (mut; Fig. 4a, middle panel). The reduction in the synaptophysin/synaptobrevin complex was not accompanied by an overall reduced amount

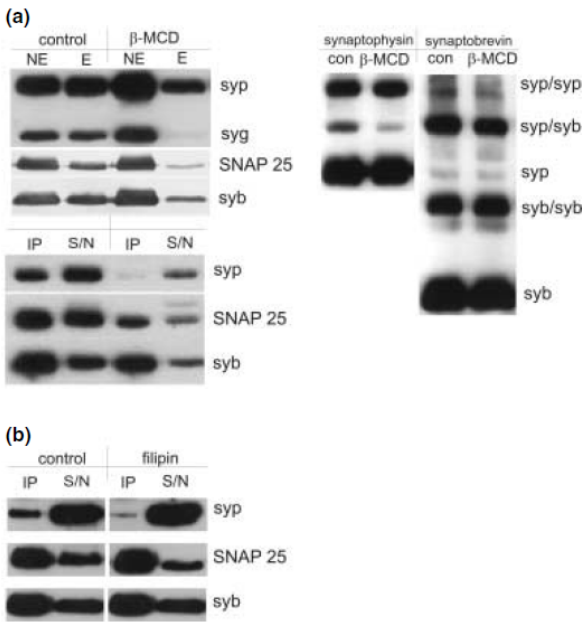

Fig. 2 Solubility of synaptophysin and its interaction with synaptobrevin in SSV from rat brain after cholesterol depletion. (a) SSV resuspended in PBS with or without 37.5 mM β-MCD were incubated at 37°C for 20 min. The reaction was stopped by either adding extraction buffer or the cross-linker to obtain a final concentration of 1% Triton X-100 or 0.5 mM DSS, respectively. Part of the supernatant of the extraction procedure was subjected to immunoprecipitation using the antibody against synaptobrevin. The rest of the extract (E) and the non-extractable material (NE) was diluted in Laemmli and comparable amounts were analysed with antibodies against synaptophysin (syp), synaptogyrin (syg), SNAP25 and synaptobrevin (syb). Similarly, immunoprecipitates (IP) and the corresponding supernatants (S/N) were analysed for the presence of synaptobrevin (syb), synaptophysin (syp) and SNAP25. The cross-linked samples were analysed using antibodies against synaptophysin and synaptobrevin. Both antibodies recognized the synaptophysin/synaptobrevin complex at 56 kDa, the synaptophysin and the synaptobrevin antibodies also stained the respective monomers and dimers. (b) SSV resuspended in PBS with or without 32.5 mM filipin were incubated at 37°C for 20 min. The reaction was stopped by adding extraction buffer followed by an immunoprecipitation with the antibody against synaptobrevin. Note that β-MCD and filipin treatment reduces the solubility of synaptophysin and its ability to complex synaptobrevin.

of a variety of synaptic proteins analysed in mutant and heterozygous littermates (Fig. 4a, right panel). For quantification, four pairs of heterozygous and homozygous mutant littermates were analysed and the ratio between synaptophysin and the synaptophysin/synaptobrevin complex was calculated when using the synaptophysin antibody, and the ratio between synaptobrevin and the complex was estimated when using the synaptobrevin antibody. Similar ratios between the respective monomers and dimers were calculated. A significant reduction was observed for the synaptophysin/synaptobrevin complex

Table 1 Effects of β-MCD on the extractability of synaptic vesicle proteins (A) and the reduction of the synaptophysin/synaptobrevin complex (B)

A Proteins in extract	Control	β-MCD
Synaptophysin	88.5 ± 7.4 ($n = 4$)	52 ± 9.2 ($n = 3$) ($^*p = 0.01$)
Synaptobrevin	70 ± 7.8 ($n = 4$)	77 ± 7.8 ($n = 4$)
Syntaxin	50 ± 4.8 ($n = 4$)	56 ± 9.7 ($n = 4$)

Crude rat synaptic vesicles (two separate samples of either 500 μg or 1 mg protein, respectively) were treated for 60 min with or without 40 mM β-MCD before they were extracted using 1% Triton X-100. The amount of protein found in the extract was quantified. Note that only the amount of synaptophysin was siginificantly reduced due to β-MCD treatment.

B Reduction of synaptophysin/synaptobrevin complex after treatment with β-MCD detected with antibodies against	
Synaptobrevin (% of control)	Synaptophysin (% of control)
39.4 ± 20.5 ($n = 5$)	42.5 ± 28 ($n = 3$)

Crude rat synaptic vesicles were treated for 60 min with or without 40 mM β-MCD before they were subjected to cross-linking using DSS. The ratio between the synaptophysin/synaptobrevin complex to the amount of the synaptobrevin monomer was calculated when using the synaptobrevin antibody and the complex was referred to synaptophysin monomer when using the synaptophysin antibody for detection, respectively. For each experiment (number given in brackets) the amount of complex is given as percentage of the complex in the respective control experiment.

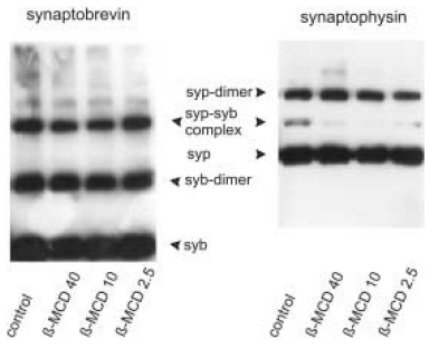

Fig. 3 β-MCD dose-dependently decreases of the synaptophysin/synaptobrevin complex in rat SSV. The experiment was performed as given in Fig. 2(a).

in homozygous mutants. The synaptophysin dimer appeared to be slightly reduced but the synaptobrevin dimer remained unchanged (Fig. 4b). As expected the overall cholesterol content in synaptosomal preparations from wild-type and NPC1 homozygous mutants was similar (not shown).

In order to further substantiate a putative correlation between an overall cholesterol content of SSV membranes to the synaptophysin/synaptobrevin complex we analysed the cholesterol content of an LP2 preparation from adult and embryonic rat brain as well as membranes from the synaptophysin-expressing cell line CHOp38. Embryonic vesicles and CHOp38 membranes contain considerable amounts of synaptophysin. In contrast to SSV membranes from adult brain this synaptophysin does not bind endogenous (LP2) or exogenously offered (LP2 and CHOp38) recombinant synaptobrevin (Becher *et al.* 1999a, 1999b). The cholesterol content in an embryonic LP2 and in membranes from CHOp38 was 97.6 nmoles/mg protein and 58.4 nmoles/mg protein, respectively, and thus from three- to five-fold lower compared to 282 nmoles/mg protein obtained with a LP2 preparation from adult rat brain. These data support the view that the vesicular cholesterol content critically influences the synaptophysin/synaptobrevin interaction.

The availability of cholesterol content appears to correlate directly with synaptophysin/synaptobrevin interaction as observed in cultured embryonic hippocampal neurons grown for 10–12 days *in vitro* (DIV) . Treatment with lovastatin, an inhibitor of HMG-CoA-reductase that prevents endogenous cholesterol synthesis, reduced the synaptophysin/synaptobrevin interaction without affecting synaptobrevin's interaction with SNAP25 (Fig. 5a). Alternatively, addition of cholesterol to hippocampal neurones increased the synaptophysin/synaptobrevin interaction almost two-fold after 11 DIV compared to the respective control cultures (Figs 5b and c).

Discussion

In the present study we have shown that depletion of cholesterol in various experimental or pathophysiological conditions reduces the solubility of synaptophysin in Triton X-100 and decreases the interaction of synaptophysin with synaptobrevin. In hippocampal cultures, cholesterol treatment increases the synaptophysin/synaptobrevin interaction.

Generally, cholesterol is an abundant lipid that is present in every membrane where it is responsible for membrane fluidity. Membranes of organelles and the plasma membrane differ in the ratio between cholesterol and phospho- or sphingolipids. SSVs are characterized by a very high cholesterol content. During neuronal development, cholesterol content increases and appears to correlate with synaptic maturation. Recently, a direct correlation between cholesterol content and synaptogenesis has been shown for retinal

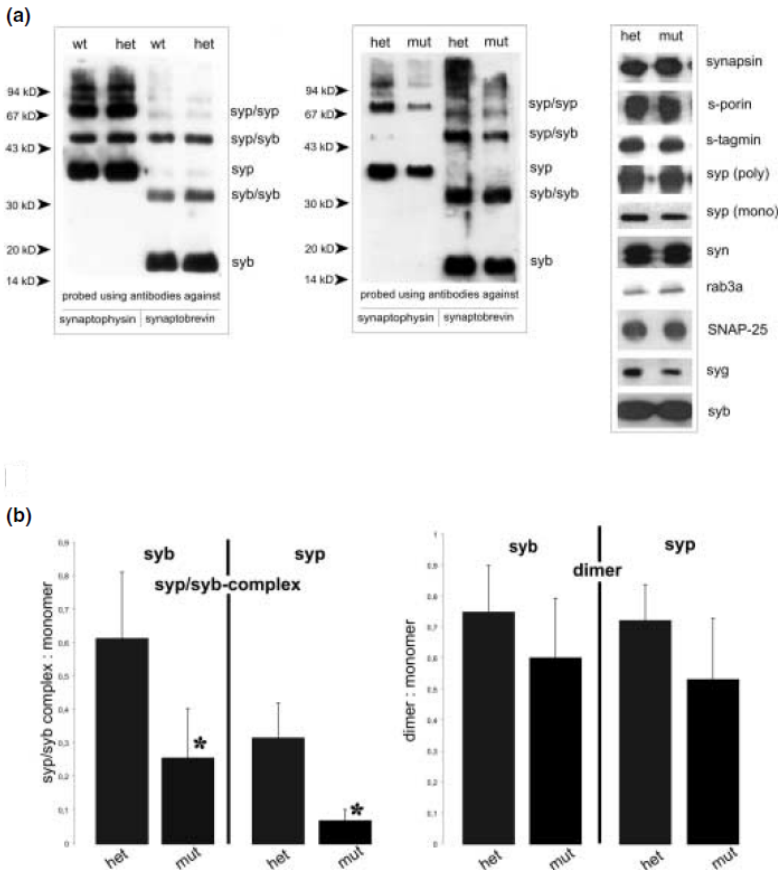

Fig. 4 The synaptophysin/synaptobrevin complex is reduced in NPC1-mutant mice. SSV of whole brains from either wild-type, heterozygous or homozygous mutants were cross-linked followed by SDS–PAGE analysis and immunoreplica using antibodies against synaptophysin and synaptobrevin. (a) No differences in the amount of synaptophysin, synaptobrevin and their respective complexes were found when comparing wild-type (wt) and heterozygous mutants (het; left panel). A clearcut reduction of the synaptophysin/synaptobrevin complex was seen when analysing homozygous (mut) mutants (middle panel). The third panel shows a great variety of synaptic proteins which occur in equal amounts in wild type and homozygous mutants. (b) SSV from four pairs of heterozygous (het) or homozygous (mut) littermates were subjected to cross-linking and then analysed using the antibodies against synaptophysin or synaptobrevin. The ratios between the synaptophysin/synaptobrevin complex (left diagram) and either synaptobrevin or synaptophysin, respectively, were found to be: control 0.62 ± 0.19, mutant 0.26 ± 0.15 ($p = 0.023$, Student's *t*-test) for detection with anti-synaptobrevin and control 0.32 ± 0.1, mutant 0.07 ± 0.03 ($p = 0.033$) for detection with anti-synaptophysin. The ratios between the dimers (right diagram) and their respective monomers were calculated to be as follows: control 0.75 ± 0.15, mutant 0.6 ± 0.19 ($p = 0.18$) for detection with anti-synaptobrevin and control 0.72 ± 0.11, and mutant 0.53 ± 0.2 ($p = 0.75$) for detection with antisynaptophysin. *Note that there is a significant decrease of the synaptophysin/synaptobrevin complex in mutant mice obtained after detection with either antibody.

ganglion cells in culture (Mauch *et al.* 2001). Cholesterol treatment also increases the synaptophysin/synaptobrevin interaction (this paper), suggesting that this complex is an indicator for synapse maturation. In addition to the endogenous synthesis in neurones, cholesterol has to be provided in great abundance by astrocytes, suggesting that developing neurones depend on exogenous cholesterol (Mauch *et al.* 2001). The fact that SSV preparations obtained from embryonic brains contain less cholesterol compared to adult SSV also supports the idea that sufficient cholesterol is required for the maturation of synaptic terminals. In contrast, the maturation of the axonal plasma membrane depends on the up-regulation of sphingomyelin (Ledesma *et al.* 1999).

Cholesterol is not evenly distributed but enriched in membrane patches referred to as detergent-resistant membranes (DRM) where specific proteins cluster. Proteins trapped in DRM become soluble in detergents only after cholesterol depletion. Besides these unusually stable DRM's, clusters containing SNARE proteins exist which can be segregated from DRMs by their solubility in detergents (Lang *et al.* 2001). These protein clusters are less stable and can be evenly dispersed in the planar membrane by the

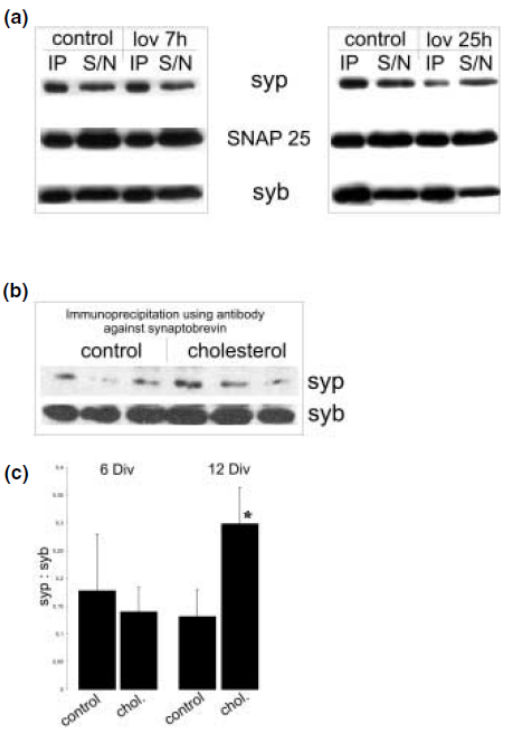

Fig. 5 Effects of cholesterol on the upregulation of the synaptophysin/synaptobrevin complex in primary cultures. (a) Hippocampal primary cultures were treated at DIV 11 for either 7 h or 25 h with 5 μM lovastatin. Neurones were harvested, extracted by Triton X-100 and subjected to immunoprecipitation using the antisynaptobrevin antibody. Immunoprecipitates (IP) and the corresponding supernatants (S/N) were analysed for synaptophysin (syp), SNAP25 or synaptopbrevin (syb). After 25-h lovastatin treatment, a reduction of synaptophysin is visible in the synaptobrevin IP. (b) Hippocampal primary cultures were treated at DIV 1 with 10 μg/mL cholesterol dissolved in ethanol. They were cultivated till DIV 11 and then subjected to extraction and immunoprecipitation as given above. Three individual cultures for each condition were analysed. The amount of synaptophysin in the synaptopbrevin IP was increased by 190% in cholesterol-treated cultures. (c) Hippocampal primary cultures were treated at DIV 1 with 20 μg/mL cholesterol dissolved in ethanol. They were cultivated till either DIV 6 ($n = 5$) or DIV 12 ($n = 4$) and then subjected to extraction and immunoprecipitation as given above. Values are the mean of two or three individual cultures obtained from two preparations (± SD). After 10 days treatment with cholesterol there was a significant ($^*p = 0.022$) increase in the amount of the synaptophysin/synaptobrevin complex.

removal of cholesterol. They are relevant for exocytosis as seen in PC12 cells and appear to be the defined and highly preferable sites for docking and fusion of secretory vesicles (Lang *et al.* 2001). In contrast to proteins in DRM or the SNARE proteins, synaptophysin is less soluble after cholesterol depletion and also appears to aggregate and therefore might represent yet another membrane specialization where a high cholesterol content is required for an even distribution of synaptophysin.

Indeed, small synaptic vesicles are characterized by a high cholesterol content and the integral membrane protein synaptophysin, both being necessary to yield the high curvature of these organelles. Synaptophysin as a cholesterol binding protein is required to maintain the integrity of synaptic vesicles during the endocytotic pathway. Following membrane fusion, synaptophysin probably prevents membrane dispersions of SSV in the plasma membrane by clustering the membrane proteins of SSV and segregating them from the plasma membrane (Röper *et al.* 2000; Huttner and Schmidt 2000; Thiele *et al.* 2000; Huttner and Zimmerberg 2001). In mature SSVs, synaptophysin appears to have an additional function, by binding to synaptobrevin it might promote the formation of functional SNARE complexes and such an interaction depends on the cholesterol content as shown in the present study for the synaptophysin/synaptobrevin interaction. The absence of the synaptophysin/synaptobrevin complex in embryonic SSV and in membrane preparations from synaptophysin-expressing CHO cells and neuroendocrine PC12 cells can be explained as a post-translational change of the synaptophysin molecule during neuronal maturation (Becher *et al.* 1999a,b). In addition a high cholesterol content may favour the synaptophysin/synaptobrevin interaction as observed in adult SSV and also in hippocampal neuronal cultures. Thus a modification in the synaptophysin molecule and a high cholesterol content appear to be necessary for its interaction with synaptobrevin. By binding to synaptobrevin, synaptophysin might generate a membrane domain on the synaptic vesicle by which an optimal alignment of reactive synaptobrevin molecules is guaranteed. Such an alignment might concentrate all available synaptobrevin molecules at that site of the spherical vesicle where fusion will occur, so most effectively promoting the interaction with the SNARE partners at the plasma membrane.

Synaptophysin is added to a growing group of cholesterol-binding proteins that include a variety of key enzymes like the HMG-CoA-reductase, transport proteins like the NPC1 protein but also the SNARE protein syntaxin (Lang *et al.* 2001). The sonic hedgehog protein shh-1 has been also identified as a cholesterol-binding protein, which, however, undergoes a post-translational covalent binding of cholesterol (Cooper *et al.* 1998). It remains to be determined whether the association of cholesterol to synaptophysin is an hydrophobic interaction or whether it involves an additional covalent binding which might up-regulate during neuronal development and be part of the crucial modification that allows synaptophysin to interact with synaptobrevin.

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“The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis.”

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The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis

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Abstract

Synaptophysin is one of the most abundant membrane proteins of small synaptic vesicles. In mature nerve terminals it forms a complex with the vesicular membrane protein synaptobrevin, which appears to modulate synaptobrevin's interaction with the plasma membrane-associated proteins syntaxin and SNAP25 to form the SNARE complex as a prerequisite for membrane fusion. Here we show that synaptobrevin is preferentially cleaved by tetanus toxin while bound to synaptophysin or when existing as a homodimer. The synaptophysin/synaptobrevin complex is, however, not affected when neuronal secretion is blocked by botulinum A toxin which cleaves SNAP25. Excessive stimulation with α-latrotoxin or Ca^{2+}-ionophores dissociates the synaptophysin/synaptobrevin complex and increases the interaction of the other SNARE proteins. The stimulation-induced dissociation of the synaptophysin/synaptobrevin complex is not inhibited by pre-incubating neurones with botulinum A toxin, but depends on extracellular calcium. However, the synaptophysin/synaptobrevin complex cannot be directly dissociated by calcium alone or in combination with magnesium. The dissociation of synaptobrevin from synaptophysin appears to precede its interaction with the other SNARE proteins and does not depend on the final fusion event. This finding further supports the modulatory role the synaptophysin/synaptobrevin complex may play in mature neurones.

Keywords: clostridial neurotoxins, neuronal stimulation, synaptobrevin, synaptophysin, SNARE complex, Syp/Syb-complex.

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Synaptophysin is one of the most abundant synaptic vesicle proteins in brain and neuroendocrine tissue. Despite this fact, little is known about its physiological role especially in neurones. Synaptophysin interacts with synaptobrevin in mature neurones (Edelmann *et al.* 1995). Synaptobrevin also forms a tight complex with the plasma membrane proteins syntaxin and SNAP25, better known as the SNARE complex, which is a prerequisite for membrane fusion (Jahn and Südhof 1999; Lin and Scheller 2000).

Increasing the amount of synaptophysin by transfection or decreasing it when using an antisense approach leads to an increase or decrease in the synaptic efficiency at the neuromuscular junction, respectively (Alder *et al.* 1992, 1995). On the other hand, synaptophysin-deletion mutants exhibit an apparently normal phenotype (Eshkind and Leube 1995; McMahon *et al.* 1996), showing that synaptophysin is not absolutely required for exocytosis. The synaptophysin/synaptobrevin complex (Syp/Syb-complex) is absent in neuroendocrine cells and embryonic neurones although both proteins reside on the same vesicle, but it is upregulated during neuronal development indicating a role in synaptic maturation (Becher *et al.* 1999a, 1999b). When comparing synaptophysin-expressing with synaptophysin-deficient neurones under competitive culture conditions, the former were proven to be better donors of pre-synaptic terminals (Tarsa and Goda 2002). Furthermore, in kindled rats which represent a model of human epilepsy and where synaptic activity is enhanced persistently by repetitive stimulation over weeks (Lothman 1997; McNamara and Wada 1997), an

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Abbreviations used: DIV, days *in vitro*; PBS, phosphate-buffered salt solution; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Syp/Syb-complex, synaptophysin/synaptobrevin complex.

increase in the amount of the Syp/Syb-complex is observed (Hinz *et al.* 2001). These findings indicate that synaptophysin, although not absolutely required, may positively modulate neuronal exocytosis.

SNARE proteins are cleaved by clostridial neurotoxins but appear to be protected when having entered the SNARE complex (Pellegrini *et al.* 1995; Otto *et al.* 1997). Whether synaptobrevin is accessible in its complex with synaptophysin is not known, and neither are the consequences for this complex when blocking neuroexocytosis by cleaving SNAP25. Assuming that the Syp/Syb-complex may help to get synaptobrevin molecules aligned in the vesicular membrane in a way to facilitate their interaction with the plasma membrane SNARE proteins syntaxin and SNAP25, synaptobrevin should be dissociated from synaptophysin following stimulation. In the present study, consequences on the Syp/Syb-complex of inhibiting or stimulating neuronal activity were investigated.

Materials and methods

Antibodies

The following antibodies were generous gifts from R. Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany): mouse monoclonal antibodies against synaptobrevin II (clone 69.1, Edelmann *et al.* 1995) and synaptophysin (clone 7.2, Jahn *et al.* 1985). Monoclonal antibodies against syntaxin1A/B (clone HPC-1) and SNAP25 were purchased from Synaptic Systems (Göttingen, Germany) or from Sternberger Monoclonals (Baltimore, MD, USA), respectively.

Secondary antibodies for western blot detection, horse anti-mouse and goat anti-rabbit conjugated with horseradish peroxidase were purchased from Vector Laboratories (Burlingame, CA, USA).

An antibody specific for the C-terminus of SNAP25 raised in rabbits (Ekong *et al.* 1997) was kindly provided by J. Frevert (Biotecon, Potsdam, Germany).

Toxins and other chemicals

Tetanus toxin and its light chain were purified as described (Weller *et al.* 1989). SLO was a generous gift from U. Weller (Labor Dr Kramer und Kollegen, Geesthacht, Germany). BoNt/A was obtained from List Biological Laboratories (Campbell, CA, USA). A-latrotoxin, the calcium ionophores A23187 and ionomycin were purchased from Calbiochem (San Diego, CA, USA).

Synaptosomes and synaptic vesicles

Isolated nerve terminals (crude synaptosomes) were prepared at 4°C from adult rat whole brains in the presence of protease inhibitors (Edelmann *et al.* 1995; Becher *et al.* 1999a). Crude synaptic vesicles (LP2 fraction) were prepared from rat brains following the procedure described (Edelmann *et al.* 1995). Synaptosomes (1–2 mg of protein) were incubated with SLO to permeabilize the plasma membrane following an addition of tetanus toxin preactivated by 10 mM dithiothreitol (DTT) for 30 min at 37°C or its light chain. For cross-linking, synaptosomal pellets were suspended in Krebs–Ringer–HEPES buffer containing: NaCl 140 mM, $NaHCO_3$ 5 mM, $MgCl_2$ 1 mM, Na_2HPO_4 1.2 mM, glucose 10 mM, HEPES-NaOH 20 mM, pH 7.4 and incubated with the chemical cross-linker disuccinimidyl suberate (DSS) dissolved in dimethyl sulfoxide, to yield a final concentration of 0.5 mM. Synaptic vesicles (0.5–1 mg protein) were incubated in phosphate-buffered salt solution (PBS) supplemented with the compounds to be tested. In some experiments a sodium buffer containing: NaCl 150 mM, HEPES-NaOH 20 mM, pH 7.4 was used. After incubation at room temperature for 45 min under rotation, the reaction was quenched by incubating for 30 min with Tris–HCl pH 7.4 (final concentration of 100 mM), membranes were subsequently pelleted at 350 000 *g* for 30 min and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting under non-denaturating conditions. In some experiments cleavage with tetanus toxin was performed after the crosslinking procedure in the presence of 0.1% Triton X-100. Protein content was measured using the BCA (bicinchoninic acid) test (Sigma, St Louis, MO, USA).

Hippocampal primary cultures

Primary cultures from embryonic (embryonic day 17) mouse hippocampal neurones were prepared and cultivated up to 14 days *in vitro* (DIV; Grosse *et al.* 2000).

Cultures were treated with tetanus or botulinum A toxin for the days indicated. For stimulation neurones were incubated in Krebs–Ringer–HEPES buffer supplemented with α-latrotoxin, the Ca^{2+}-Ionophores A23187 or ionomycin for 20 min at 37°C. Incubation was stopped by placing the culture dishes on ice and neurones were suspended in PBS and lysed by adding nine parts of water supplemented with protease inhibitors [pepstatin/leupeptin and phenylmethylsulphonyl fluoride (PMSF)]. This suspension was first homogenized (nine strokes 900 rpm) and then subsequently passed nine times through a 23G and six times through a 27G needle followed by centrifugation at 12 000 *g* to discard the nuclear debris. The supernatant was centrifuged for 30 min at 220 000 *g* and the membranes were finally subjected to the extraction procedure. In some experiments scraped neurones were centrifuged at 1000 *g* for 5 min followed by an extraction and immunoprecipitation (see below) which gave similar results.

Immunoprecipitation

Extraction was performed with 0.2–1 mL extraction buffer (about 1–1.5 mg protein/mL) containing: KCl 140 mM, EDTA 2 mM, HEPES-KOH 20 mM, pH 7.3 and 1% (v/v) Triton X-100 for 1 h at 4°C under rotation, followed by centrifugation for 3 min at 700 *g*. 2.5 µL/mL extract of ascites fluid (corresponding to about 7.5 µg of IgG) of the monoclonal antibodies against either synaptobrevin, synaptophysin or syntaxin were added to 200 µL of extract. Incubation was carried out for 16–18 h at 4°C. Immunoprecipitates were separated by addition of 25 µL of G-Sepharose Fast Flow beads (Pharmacia Biotech, Piscataway, NJ, USA). The beads were then pelleted for 1 min at 200 *g* and washed three times in extraction buffer. Finally, the beads were resuspended in sample buffer, boiled for 5 min and then analysed on SDS–PAGE followed by western blotting. The supernatant from the immunoprecipitation was analysed in parallel.

ECL-processed films were scanned by video imaging and protein bands were densitometrically quantified using the SCAN PACK 3.0 program. Quantification after immunoprecipitation was performed

as described (Hinz *et al.* 2001) along with statistical analysis using the paired Student's *t*-test type 3.

Results

As a first aspect, we investigated whether synaptobrevin is protected or preferentially cleaved by tetanus toxin when binding to synaptophysin. As expected, synaptobrevin completely disappeared when cultivating mouse hippocampal neurones for 17 days in the presence of tetanus toxin, whereas the expression of synaptophysin and the interaction between SNAP25 and syntaxin were unaltered (Fig. 1a). For a differential analysis of the synaptobrevin bound to synaptophysin, synaptosomes from rat brain were permeabilized with SLO followed by an incubation with either pre-activated tetanus toxin or the tetanus toxin light chain for 30 min at 37°C, before the cross-linking of proteins by DSS. The antibody against synaptobrevin clearly recognized the Syp/Syb-complex at 56 kDa and the synaptobrevin homodimer at 36 kDa in the control sample. After tetanus toxin treatment, approximately 80% of the synaptobrevin monomer compared to control was still present, whereas synaptobrevin as homodimer was reduced to 50% and no longer detectable in its complex with synaptophysin, which was unchanged (Fig. 1b). Synaptobrevin bound to synaptophysin or homodimerized was also accessible to tetanus toxin light chain when cleavage followed the cross-linking procedure. The Syp/Syb-complex was only present after cross-linking and was absent in the tetanus light chain-treated samples as seen by the antibodies against synaptobrevin (Fig. 1c, left upper panel) or synaptophysin (Fig. 1c, left lower panel). The broader band seen by the antisynaptobrevin antibody after cross-linking might represent the c subunit of the vacuolar proton pump, a 10 kDa protein, that has been shown to interact with synaptobrevin (Galli *et al.* 1996). Incubating cross-linked crude synaptic vesicles with tetanus toxin light chain at 1 or 0.2 μM further indicated the greater sensitivity of synaptobrevin in the two complexes towards toxin cleavage. The amount of synaptobrevin detectable either in the Syp/Syb-complex or as homodimer is reduced to approximately 34% and 40% of control after incubation with 1 μM or 0.2 μM light chain, respectively, while the monomer was only reduced to 76% and 83% (Fig. 1c, right panel and legend). Thus, synaptobrevin is preferentially cleaved in its homodimeric form and in the complex with synaptophysin.

Next we analysed the Syp/Syb-complex in hippocampal neurones treated with botulinum A toxin. This toxin should not interfere with synaptobrevin but like tetanus toxin completely blocks exocytosis. BoNt/A between 12 and 100 pM completely cleaved SNAP25 as can be seen from the small shift in the electrophoretic mobility when using the SNAP25 antibody. Cleavage could be better appreciated by an antiserum raised against a peptide mimicking the newly generated C terminal part (SNAP25 p1–16) of SNAP25. This

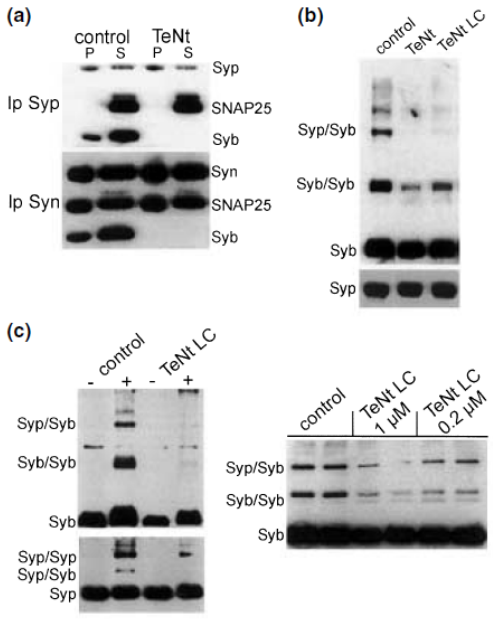


Fig.1 (a) Hippocampal neurones were treated at day *in vitro* (DIV) 7 with or without 1 nM tetanus toxin and further cultivated up to DIV 18. Neurones were harvested in PBS and collected by rapid centrifugation. Pellets were subjected to the extraction and immunoprecipitation procedure using antibodies against either synaptophysin (Ip Syp) or syntaxin (Ip Syn). The immunopreciptiates (P) and the corresponding supernatans (S) were probed with the antibodies indicated. Note that the synaptobrevin is preferentially cleaved in its homodimeric form (Syb/Syb) and in the complex with synaptophysin. The complete absence of synaptobrevin does not prevent the interaction of syntaxin with SNAP25. (b) SLO-permeabilized synaptosomes were incubated with pre-activated tetanus toxin (TeNt, final concentration 660 nM) or the tetanus toxin light chain (TeNt LC, final concentration 22 nM) for 30 min at 37°C in PBS. The synaptosomes were collected by centrifugation and subjected to the cross-linking procedure. After SDS–PAGE and transfer to nitrocellulose the blots were analysed using an antibody against synaptobrevin or synaptophysin. The amount of synaptobrevin monomer in tetanus toxin-treated samples was 76% and that in samples treated with the light chain 87%. While synaptobrevin bound to synaptophysin was no longer visible, its amount in the dimer was decreased to 50% or 53% in tetanus toxin- or the tetanus toxin light chain-treated samples, respectively. The amount of synaptophysin analysed in parallel was not changed. (c, left side) Synaptosomes either subjected to cross-linking (+) or incubated in the absence of DSS (–) before they were digested by 2 μM TeNt LC. Syb/Syb and Syp/Syp denote the synaptobrevin or synaptophysin homodimer, respectively. (c, right side) Crude synaptic vesicles LP2 were subjected to cross-linking before they were incubated with buffer (control) or either 1 μM or 0.2 μM TeNt LC, respectively. Blot strips were analysed by the synaptobrevin antibody. The following values as percentage of control were obtained: synaptobrevin monomer 76.2% or 82.8%, dimer 32.8% or 40%, and the Syp/Syb-complex 43.2% or 40.2% after 1 μM or 0.2 μM TeNt LC, respectively. The values represent the mean of two independent samples.

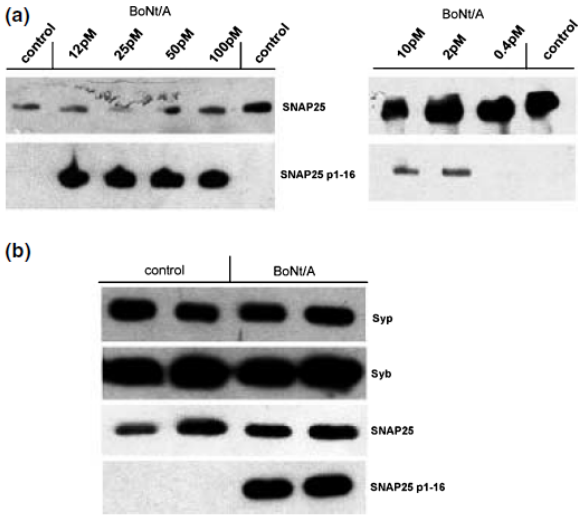
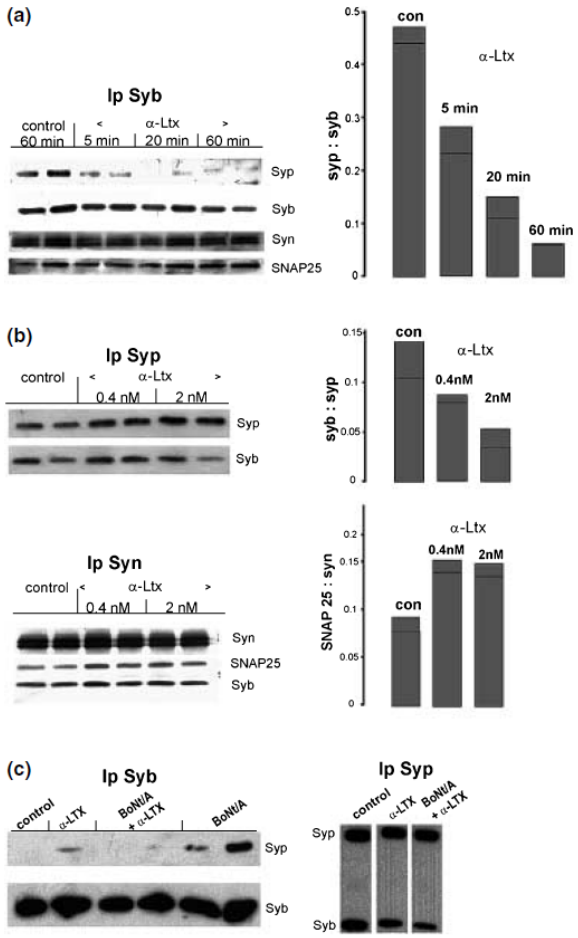


Fig. 2 BoNt/A has no effects on the Syp/Syb-complex. (a) Hippocampal neurones were treated at DIV 6 with the BoNt/A concentrations indicated or without (two independent controls at the left and the right side of the blot) and cultivated up to DIV 14. SNAP25 was analysed using either the usual antibody (SNAP25) or an antiserum against the newly generated C-terminal part of SNAP25 (SNAP25 p1–16). Cleavage was also discernible at 2 pM BoNt/A-treatment (right panel). (b) Hippocampal neurones were treated with or without BoNt/A at DIV 6 and cultivated up to DIV 14 before they were subjected to the extraction and immunoprecipitation procedure using an antibody against synaptobrevin. Immunoprecipitates were analysed using the antibodies indicated at the right side. Experiment was performed in duplicates with two sets of control cultures and two treated with BoNt/A. Note that a small shift was visible using the SNAP25 antibody whereas the SNAP25 p1–6 antiserum only detects the cleaved SNAP25 in the BoNt/A-treated but not in control cultures. Note that the Syp/Syb-complex is not affected by BoNt/A-treatment.

antiserum did not recognize full-length SNAP25 which was only present in control cultures (Fig. 2a). Using this antibody cleavage was discernible after treatment with 2 pM BoNt/A (Fig. 2a, right panel). Treatment of hippocampal neurones with BoNt/A did not interfere with the Syp/Syb-complex analysed by immunoprecipitation using the synaptobrevin antibody (Fig. 2b). The shortened SNAP25 in the BoNt/A-treated neurones was also immunoprecipitated by the synaptobrevin antibody, indicating that it still was able to bind to synaptobrevin (Fig. 2b).

To test whether excessive stimulation may dissociate the Syp/Syb-complex we applied α-latrotoxin to mature (DIV 14) hippocampal neurones for 5 and 60 min 1 nM α-latrotoxin caused a time-dependent decrease in the amount of the Syp/Syb-complex (Fig. 3a), while the interaction between synaptobrevin and SNAP25 analysed in parallel increased slightly (not shown). Similar, when using an antibody against synaptophysin for immunoprecipitation, α-latrotoxin stimulation for 20 min dose-dependently decreased the



Syp/Syb-complex while increasing the association between SNAP25 and syntaxin as visualized by an immunoprecipitation using an antibody against syntaxin (Fig. 3b). Pretreatment of hippocampal neurones with BoNt/A for 8 days did not prevent the α-latrotoxin-induced dissociation of the Syp/Syb-complex in DIV 14 hippocampal neurones (Fig. 3c). The stimulation-dependent dissociation of the Syp/Syb-complex also occurred after treatment with the Ca^{2+} ionophore A23187 for 20 min (Fig. 4a). The amount of Syp/Syb-complex decreased between 50 and 70% compared to control cultures (Fig. 4b). A decrease was also observed when stimulating BoNt/A-treated neurones; however, for unknown reasons, the variability was greater in these samples (Figs 4a and b). These data suggest that the dissociation of the Syp/Syb-complex does not depend on the final membrane fusion and may precede synaptobrevin's entering the SNARE complex.

As another aspect we analysed the Ca^{2+}-dependency of the stimulated dissociation of the Syp/Syb-complex using the Ca^{2+}-specific ionophore ionomycin. Hippocampal neurones (21 DIV) were stimulated for 20 min at 37°C with 10 μM ionomycin dissolved either in normal KR–HEPES or in

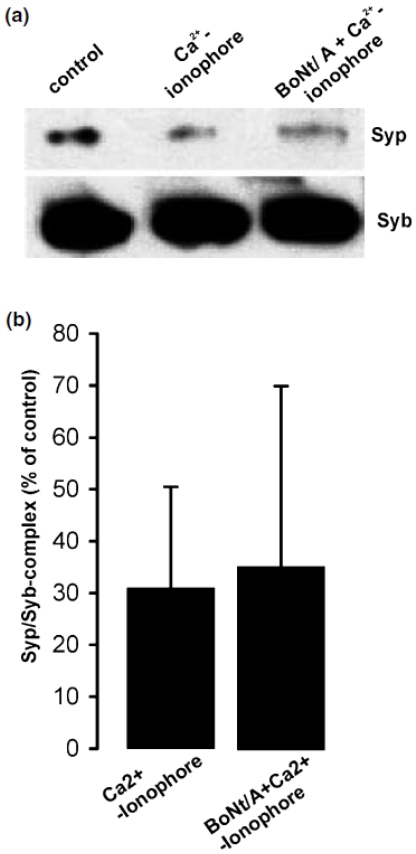


Fig. 3 Stimulation with α-latrotoxin dissociates the Syp/Syb-complex. (a) Hippocampal neurones (DIV 14) were incubated with 1 nM α-latrotoxin dissolved in KR–HEPES buffer for the indicated time periods before they were collected and subjected to the extraction and immunoprecipitation procedure using an antibody against synaptobrevin. Immunoprecipitates were analysed using the antibodies indicated at the right side. The experiment was performed in duplicate and the ratio between synaptophysin and synaptobrevin was quantified. The individual values are indicated by the bars in the diagram. The amount of Syp/Syb-complex decreased depending on the time of incubation with α-latrotoxin. (b) Hippocampal neurones (DIV 14) were incubated with α-latrotoxin dissolved in KR–HEPES buffer at the concentrations indicated for 20 min at 37°C, before extracting and immunoprecipitating them using antibodies against either synaptophysin (Ip SyP) or syntaxin (Ip Syn). α-Latrotoxin treatment dose-dependently decreased the Syp/Syb-complex while increasing the interaction between SNAP25 and syntaxin. Experiment was run in duplicate. (c) Hippocampal neurones were treated with BoNt/A 130 pM at DIV 6 and cultivated up to DIV 14. Naive and toxin-treated neurones were stimulated with 1 nM α-latrotoxin for 20 min at 37°C. After extraction, immunoprecipitation was performed with an antibody against synaptobrevin (left panel) and blot strips were analysed for synaptophysin and synaptobrevin. Whereas the synaptophysin/synaptobrevin complex was clearly visible in control and BoNt/A-treated neurones, it could not be detected when cultures were stimulated with α-latrotoxin. In a parallel experiment, antibody against synaptophysin instead of against synaptobrevin was used for immunoprecipitation (right panel). The amount of complex compared to controls was either 57% or 62% after α-latrotoxin alone or after α-latrotoxin in the presence of BoNt/A, repectively. Note that BoNt/A treatment does not overcome the dissociation of the Syp/Syb-complex due to α-latrotoxin.

KR–HEPES without Ca^{2+} containing EGTA. Cells were collected on ice, extracted with 1% Triton X-100 and the extracts were subjected to an immunprecipitation using the synaptobrevin antibody. As can be seen in Fig. 5(a), the dissociation of the Syp/Syb-complex was prevented when omitting calcium. A Ca^{2+}/Mg^{2+}-dependent dissociation of the Syp/Syb-complex in Triton extracts from rat synaptosomes had already been described (Prekeris and Terrian 1997; Daly and Ziff 2002). In order to see a direct interaction between Ca^{2+} elevation and the Syp/Syb-complex, we incubated synaptic vesicles (LP2) with EGTA, Ca^{2+} alone, or Ca^{2+} in combination with Mg^{2+} for 30 min at 37°C, followed by cross-linking. The detection by a synaptobrevin antibody revealed no dissociation of the Syp/Syb-complex after incubation with either Ca^{2+} alone, or Ca^{2+} in combination with Mg^{2+}. Incubation of synaptic vesicles with a Ca^{2+}/Mg^{2+} combination in the presence of ATP also did not dissociate the Syp/Syb-complex analysed by immunoprecipitation using the synaptophysin antibody, whereas the complex reversibly dissociated after high salt treatment or reduction (Fig. 5c). Even when treating synaptic vesicles with a combination of Ca^{2+} and Mg^{2+} in the presence of Triton, thereby using the protocol of Daly and Ziff (2002), no dissociation could be observed. Thus the Syp/Syb-complex

Fig. 4 Stimulation with the Ca^{2+} ionophore dissociates the Syp/Syb-complex in a BoNt/A-resistant manner. (a) Hippocampal neurones were treated with BoNt/A 130 pM at DIV 6 and cultivated up to DIV 14. Control and toxin-treated neurones were stimulated with or without 2 μM A23187 for 20 min at 37°C. After extraction, immunoprecipitation was performed with an antibody against synaptobrevin and blot strips were analysed for synaptophysin and synaptobrevin. (b) The ratio between synaptophysin and synaptobrevin was quantified and the amount of the complex expressed as percentage of control (0.64 ± 0.2; $n = 6$) shown in the diagram. Data represent the mean of either six or four samples for ionophore or ionophore after BoNt/A-treatment, respectively, obtained from four different preparations. In detail, the following values were obtained: Experiment 1: A23187, 30% and 33%; BoNtA plus A23187, 35% and 19%. Experiment 2: A23187, 34% and 12%; Experiment 3: A23187, 12%; BoNtA plus A23187, 4%; Experiment 4: A23187, 65%; BoNtA plus A23187, 85%. Note that BoNt/A treatment does not affect the decrease in the amount of the Syp/Syb-complex due to stimulation with the ionophore. For unknown reasons, variations in stimulated BoNt/A-treated cultures were greater compared to stimulated non-treated cultures.

does not appear to be dissociated by Ca^{2+} directly, but its dissociation requires a Ca^{2+}-dependent step, as revealed in our cell culture experiments, and which still might work in synaptosomal extracts (Prekeris and Terrian 1997; Daly and Ziff 2002).

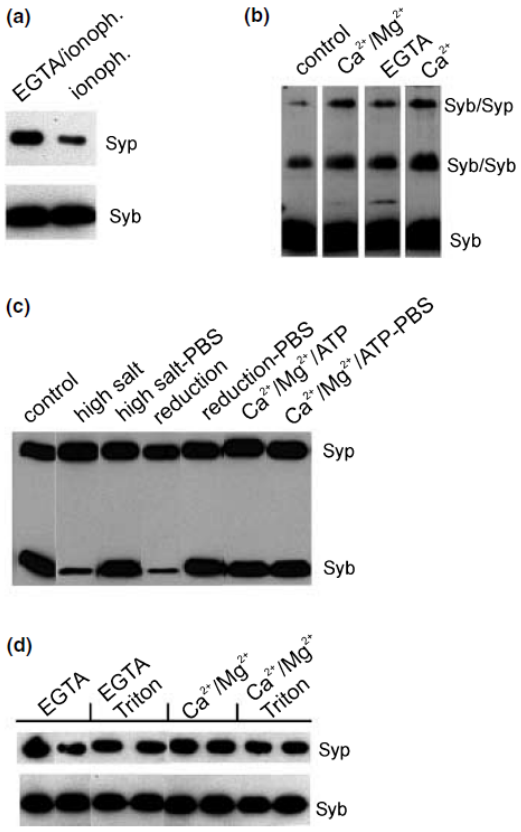


Fig. 5 Effects of Ca^{2+} on the dissociation of the Syp/Syb-complex. (a) Hippocampal neuronal cultures (DIV 21) were incubated for 20 min at 37°C in KR–HEPES buffer containing 10 μM ionomycin either in the absence (no Ca^{2+}, 2 mM EGTA) or the presence of 2.5 mM Ca^{2+}. Then cells were scrapped on ice and centrifuged down at 1000 *g* for 5 min. The pellet was subjected to the extraction and immunoprecipitation procedure using the synaptobrevin antibody. Immunoprecipitates were separated by SDS–PAGE transferred to nitrocellulose and analysed with the antibodies against synaptophysin or synaptobrevin. (b) Crude synaptic vesicles (LP2) were incubated in sodium buffer either in the absence (control) or the presence of either 1 mM $CaCl_2$ plus 2.5 mM $MgCl_2$, 2 mM EGTA, or 1 mM $CaCl_2$ alone for 30min at 37°C. After cross-linking, samples were analysed by immunoreplica using the synaptobrevin antibody. No dissociation was observed in samples treated with calcium either in the absence or presence of magnesium. (c) Crude synaptic vesicles (LP2) were incubated in PBS containing additional 450 mM NaCl (high salt) or 10% β-mercaptoethanol (reduction) or a mixture of 0.1 mM $CaCl_2$, 2.5 mM $MgCl_2$ and 1 mM ATP (Prekeris and Terrian 1997) and incubated for 30 min at room temperature. The incubation mixture was removed and one part of each sample was resuspended in PBS and further incubated for 30 min at room temperature before extraction while the other part and the controls were extracted immediately. Triton extracts were subjected to the immunoprecipitation procedure using the synaptophysin antibody and immunoprecipitates were analysed for synaptophysin and synaptobrevin content. While high salt condition or reduction caused a reversible dissociation of the Syp/Syb-complex the Ca^{2+}/Mg^{2+} treatment had no effect. (d) Synaptic vesicles (LP2) were treated with the Ca^{2+}/Mg^{2+} mixture as described in (b) either before extraction or in the presence 1% Triton (Daly and Ziff 2002). Immunoprecipitation was performed with the synaptobrevin and blots were analysed by the antibodies indicated. Again no dissociation of the Syp/Syb-complex was observed.

Discussion

In the present study we have shown that the Syp/Syb-complex disappeared after treatment with tetanus toxin, indicating that synaptobrevin when bound to synaptophysin is well available to tetanus toxin cleavage, whereas SNAP25 cleavage did not affect this complex. During excessive stimulation the Syp/Syb-complex dissociated. The dissociation was not blocked by BoNt/A but depended on a Ca^{2+}-dependent step.

Tetanus toxin-treated cultures developed normally (Ahnert-Hilger *et al.* 1996) and formed syntaxin/SNAP25 complexes in the absence of detectable synaptobrevin. Inhibiting exocytosis by BoNt/A did not interfere with the Syp/Syb-complex or its developmental upregulation in hippocampal cultures. Similar data were obtained when treating hippocampal neurones with tetrodotoxin (Bacci *et al.* 2001 and data not shown). The cleaved SNAP25 is still able to form a complex with syntaxin and synaptobrevin, although this will not lead to vesicular fusion under physiological conditions (Xu *et al.* 1998). By contrast, when glutamate receptors are blocked during the whole cultivation time, a downregulation of the Syp/Syb-complex was observed while miniature EPSC frequency was increased (Bacci *et al.* 2001). Thus, pre-synaptic vesicular release appeared not to affect the development of mature synapses, whereas blocking the post-synaptic detection system had severe effects on the maturation of pre-synaptic vesicles. Probably the blockade of NMDA receptors started at DIV 1 in culture prevented pre-synaptic terminals from forming mature synapses by locking synaptic vesicles in an immature stage (Bacci *et al.* 2001), thereby inhibiting the developmental upregulation of the interaction between synaptophysin and synaptobrevin (Becher *et al.* 1999b; Bacci *et al.* 2001). The Syp/Syb-complex is increased in repetitively stimulated (kindled) rats (Hinz *et al.* 2001), indicating that mature synapses benefit from this complex, in that it may speed up vesicular membrane fusion. How this upregulation might occur and the nature of the post-translational changes in the synaptophysin molecule are still obscure. Given the fact that a full SNARE complex can form when using synaptobrevin lacking the transmembrane domain (Sutton *et al.* 1998), but truncated synaptobrevin cannot bind synaptophysin (Becher *et al.* 1999a), it is tentative to speculate that the interaction between synaptophysin and synaptobrevin occurs in the transmembrane domains.

The putative modulatory role the Syp/Syb-complex may play in mature neurones is underscored by the fact that synaptobrevin dissociated from synaptophysin after stimulation with either α-latrotoxin or Ca^{2+} ionophores. Simultaneously, a slight increase in the interaction of the SNARE proteins was observed.

It appears that synaptobrevin is directly recruited from the synaptophysin/synaptobrevin complex. Dissociation occurs before fusion as it is also seen in BoNt/A-treated cultures. A dissociation of synaptobrevin from synaptophysin preceding membrane fusion can also be observed in α-latrotoxin-stimulated hippocampal neurones using FRET analysis (Pennuto *et al.* 2002). Short stimulation with elevated potassium concentrations, however, failed to produce a significant dissociation of the Syp/Syb-complex (data not shown), suggesting that under these conditions only part of the vesicle pool is addressed. The excessive stimulation observed after treatment with α-latrotoxin or with the Ca^{2+} ionophores may recruit all synaptic vesicles available, which in turn allows discrimination of the dissociation of synaptobrevin from synaptophysin by FRET analysis (Pennuto *et al.* 2002) or by immunoprecipitation (this paper).

The dissociation of the Syp/Syb-complex depends on an elevation of the intracellular Ca^{2+} concentration as revealed in our culture experiments after stimulation with the inophore. However, the Syp/Syb-complex cannot be dissociated directly when incubating native synaptic vesicles with Ca^{2+} under various conditions. In these vesicles, the Syp/Syb-complex dissociates upon high salt and reduction of disulphide bonds and reassembles when the reagents are removed, indicating its functionality. The Ca^{2+} insensitivity of the Syp/Syb-complex in synaptic vesicles shown under various experimental conditions contrasts with findings on synaptosomal extracts where the complex could be dissociated by Ca^{2+} (Prekeris and Terrian 1997; Daly and Ziff 2002). Because the only difference between the experiments in the literature and our approach is the use of synaptosomal extracts compared to native synaptic vesicles, it may be that the former contain compounds which in a Ca^{2+}-dependent way dissociate the complex. Thus, an elevation of the intracellular Ca^{2+} concentration does not directly dissociate the Syp/Syb-complex but requires a cytosolic factor, which is present in synaptosomal extracts (Prekeris and Terrian 1997; Daly and Ziff 2002) and cultivated neurones (this paper).

Taken together, the data presented further support the modulatory role the Syp/Syb-complex complex may play in mature neurones.

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“Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates.”

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Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates

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Abstract

Multi-domain bacterial protein toxins are being explored as potential carriers for targeted delivery of biomolecules. Previous approaches employing isolated receptor binding subunits disallow entry into the cytosol. Strategies in which catalytic domains are replaced with cargo molecules are presumably inefficient due to co-operation of domains during the endosomal translocation step. Here, we characterize a novel transport vehicle in which cargo proteins are attached to the amino terminus of the full-length botulinum neurotoxin type D (BoNT/D). The intrinsic enzymatic activity of the neurotoxin allowed quantification of the efficacy of cargo delivery to the cytosol. Dihydrofolate reductase and BoNT type A (BoNT/A) light chain (LC) were efficiently conveyed into the cytosol, whereas attachment of firefly luciferase or green fluorescent protein drastically reduced the toxicity. Luciferase and BoNT/A LC retained their catalytic activity as evidenced by luciferin conversion or SNAP-25 hydrolysis in the cytosol of synaptosomes, respectively. Conformationally stabilized dihydrofolate reductase as cargo considerably decreased the toxicity indicative for the requirement of partial unfolding of cargo protein and catalytic domain as prerequisite for efficient translocation across the endosomal membrane. Thus, enzymatically inactive clostridial neurotoxins may serve as effective, safe carriers for delivering proteins in functionally active form to the cytosol of neurones.

Keywords: dihydrofolate reductase, fusion protein, green fluorescent protein, luciferase, neuronal transporter protein, recombinant botulinum neurotoxin.
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Bacterial protein toxins represent the causative agents of many fatal human and animal diseases, e.g. cholera, diphtheria, tetanus, botulism. Most of these toxins exhibit a modular multi-domain structure. In general, one of them assures selective binding to the target tissue. The second domain bears an enzymatic activity that affects an intracellular target, in many cases components of the protein biosynthesis machinery or the adenylate cyclase complex, thereby destroying the host cell or transiently blocking specialized functions such as exocytosis in nerve endings (Middlebrook and Dorland 1984). The third domain guarantees escape of the enzymatically active domain from the endosomal compartment or the endoplasmic reticulum into the cytosol following receptor-mediated endocytosis. This translocation either occurs subsequent to acidification of the lumenal milieu by vesicular H^+-ATPases that triggers a conformational change of the toxin molecule or via interaction with the sec-61p transport complex (e.g. Williamson and Neale 1994; Schmitz *et al*. 2000).

Because of these unique abilities, several protein toxins have been extensively explored as putative transportation vehicles for the delivery of biomolecules to predetermined target tissues. Approaches aimed, for example, at the compensation of missing enzymatic activities such as in

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Abbreviations used: Baf A1, Bafilomycin A1; BoNT, botulinum neurotoxin; CNTs, clostridial neurotoxins; DHFR, dihydrofolate reductase; DIV, days *in vitro*; GFP, green fluorescent protein; HC, heavy chain; LC, light chain; LC/A, LC/D, light chain of BoNT/A or D; Luc, firefly luciferase; MPN, mouse phrenic nerve; Mtx, methotrexate; scBoNT, single chain BoNT; SNARE, soluble NSF attachment protein receptor; TeNT, tetanus neurotoxin.

lysosomal storage diseases (Dobrenis *et al*. 1992), treatment of oxidative injury (Francis *et al.* 1995), blockade of apoptosis (Liu *et al.* 1999, 2001), induction of apoptosis (Ichinose *et al.* 2002), or inhibition of neurotransmitter secretion (Duggan *et al*. 2002). In several studies, a cargo molecule was directly coupled with the isolated receptor binding domain, thus disallowing cytosolic delivery, which in most cases is, however, requisite for successful treatment. Also, attachment of cargo molecules to genuine cell binding-translocation units may not always be sufficient for cytosolic delivery (Goodnough *et al*. 2002). This is presumably due to potential co-operation between the enzymatic subunit and the translocation domain or participation of the enzymatic subunit in the translocation process via membrane interaction (Kamata *et al.* 1994; Tortorella *et al*. 1995).

To deliver cargo proteins to the cytosol of neurones, full-length clostridial neurotoxins (CNTs), whose metalloprotease activity is deactivated by mutation of key residues in the catalytic centre, may therefore be promising tools (Li *et al*. 2001). Botulinum neurotoxin type D (BoNT/D) acts with extraordinary target cell selectivity in minute quantities. It is one of seven serologically distinct serotypes of BoNTs (type A–G) that together with tetanus neurotoxin (TeNT) build the group of CNTs. The family members are produced by several species of the genus *Clostridium*, as 150-kDa single chain (sc) proteins that are subsequently cleaved either by intrinsic or host proteases to yield a ~100 kDa heavy chain (HC) encompassing the cell binding and translocation units and a ~50 kDa enzymatic light chain (LC; Montecucco and Schiavo, 1994). The LC remains connected to the HC via the translocation domain by a single disulphide bridge, non-covalent interactions and a polypeptide segment derived from the translocation unit wrapped around the LC. This structural arrangement argues for pursuing a strategy employing full-length neurotoxin, predicting that the intact molecule may act more efficiently in cytosolic delivery of cargo proteins than isolated HC, as well as low expression levels of recombinant HCs in *Escherichia coli* (A. Rummel, unpublished findings). The carboxyl-terminal cell binding domain, the H_C-fragment, exhibits high selectivity for neurones, in particular for those of the cholinergic system (reviewed in Wellhöner 1992; Bigalke and Shoer 2000). Cell recognition is primarily mediated by complex gangliosides that are enriched in neuronal cell surfaces, but proteins were postulated to be involved in productive cell binding as well (reviewed in Montecucco 1986). Glycosylphosphatidylinositol-anchored glycoproteins of lipid rafts were recently identified as such a co-receptor for TeNT in neurones (Herreros *et al*. 2001; Munro *et al*. 2001). Likewise, synaptotagmins were demonstrated to act as co-receptors for BoNT/B and BoNT/G (Nishiki *et al*. 1994; Dong *et al*. 2003; Rummel *et al*. 2004). Translocation of the LC still represents a poorly understood step in the intoxication process of CNTs. According to electron microscopy data, the translocation domain integrates into the endosomal membrane (Schmid *et al*. 1993) and forms pores of yet unresolved stoichiometry, permitting the LC to traverse the membrane. The LC then acts as zinc-endoprotease, cleaving with a serotype inherent specificity certain members of the three SNARE (soluble NSF attachment protein receptor) protein families (reviewed in Montecucco and Schiavo 1994; Niemann *et al*. 1994), presumably subsequent to disconnection of the interchain disulphide bridge by the cellular redox system (Kistner and Habermann 1992). Substrate hydrolysis finally evokes blockade of neurotransmitter release in nerve endings that persists for several weeks, depending on the serotype (Johnson 1999; Brashear *et al*. 2000), and causes the symptoms of botulism or tetanus, respectively.

To test the ability of full-length BoNTs as potential cytosolic delivery vehicles for neurones, we fused various proteins to the amino terminus of full-length BoNT/D and assessed their delivery by means of the intrinsic activity of LC/D utilizing the mouse phrenic nerve (MPN) toxicity assay and substrate cleavage in rat brain synaptosomes and primary hippocampal nerve cell cultures.

Materials and methods

Materials

Luciferin solution and luciferase were purchased from Becton Dickinson GmbH (Heidelberg, Germany), methotrexate (Mtx) and Bafilomycin A1 (Baf A1) from Merck Biosciences GmbH (Schwalbach, Germany).

Plasmid constructions

The plasmid pBoNT/D for prokaryotic expression of the full-length BoNT/D was generated by PCR using suitable primers and earlier constructed plasmids containing bacteriophage specific DNA fragments as templates (Binz *et al*. 1990). Cleaved PCR-fragments were assembled in the pASK-IBA3 vector (IBA GmbH, Göttingen, Germany), whose multiple cloning site was previously modified. A corresponding construct harbouring the mutation E230A in the catalytic domain was generated using the primer 5′-GTATGGATCCAGTAATAGCTTTAATGCATGCGTTAACACATTC-3′. Composite genes consisting of the open reading frames of BoNT/D combined with green fluorescent protein (GFP), firefly luciferase, dihydrofolate reductase (DHFR), or LC/A were created by PCR employing suitable oligonucleotide primers, the template plasmids pAM500 (Flick and Hobom 1999), pBPCLuc0 (Kovacs and Mettenleiter 1991), pDHFR (von Ahsen *et al*. 2000) or pBN3 (Vaidyanathan *et al*. 1999), and pBoNT/D as parental vector. Nucleotide sequences of all constructs were verified by DNA sequencing.

Production of recombinant proteins

Single chain BoNT (scBoNT/D) and its fusion proteins were produced utilizing the *E. coli* strain TG1 during 4 h of induction at 21°C and purified on StrepTactin sepharose beads (IBA GmbH) according to the manufacturer's instructions. Fractions containing the desired proteins were pooled, frozen in liquid nitrogen and then

kept at −70°C. For some experiments, scBoNT/D or GFP-scBoNT/D were nicked in a preparative scale between LC and HC, applying trypsin immobilized on agarose beads (Sigma-Aldrich, Munich, Germany; 0.002 U/μg BoNT) during 1 h of incubation at 30°C and vigorous shaking. Proteolytic activation proved to be complete. Appreciable amounts of degradation of the resulting HC and LC or GFP-LC were not detectable by Coomassie blue staining. For analytical analysis of trypsin sensitivity, 8 μg of the various scBoNT/D fusion proteins were incubated with 2 ng of trypsin (Sigma-Aldrich) for 5, 15, 40, or 60 min at 30°C. Samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (10% gels) and Coomassie blue staining.

Binding to rat brain synaptosomes

Synaptosomes were obtained from Wistar rats according to the protocol of Jones and Matus (1974) as detailed earlier (Rummel *et al*. 2003) and finally resuspended in physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 10 mM glucose, 0.5% bovine serum albumin, pH 7.4), with the final synaptosomal protein concentration adjusted to 10 mg/mL.

Binding assays were performed in a total volume of 30 μL physiological buffer supplemented with bovine serum albumin (0.1%) for 60 min at 0°C, employing 100 μg synaptosomal protein and a 200 nM concentration of individual scBoNT/D fusion proteins. After incubation, synaptosomes were collected by centrifugation (5000 *g*; 5 min, 4°C) and unbound material in the supernatant fraction was discarded. Pellet fractions were washed two times each with 100 μL of physiological buffer/bovine serum albumin, dissolved in sodium dodecyl sulfate sample buffer [60 mM Tris-HCl, pH 6.75, 5% (v/v) β-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 0.007% (w/v) bromphenol blue] and boiled. Samples were then analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblot. The bound BoNT/D fusion proteins were finally visualized via their carboxyl-terminal StrepTag using StrepTag specific antiserum (IBA GmbH) and enhanced chemiluminescence. Quantification was done using a Kodak image station 440cf applying the Kodak 1D v3.5.5SB program (Kodak GmbH, Stuttgart, Germany). Assays without synaptosomes or without fusion protein were run in parallel in order to control for unspecific interaction of fusion proteins and the StrepTag antiserum. Control values were subtracted from values of respective samples.

In vitro cleavage of isolated synaptobrevin

Radiolabelled rat synaptobrevin 2 was synthesized *in vitro* from a pSP72-derived plasmid using the reticulocyte lysate system (Promega GmbH, Mannheim, Germany) and L-[^{35}S]methionine (555 kBq, >37 TBq/mmol; Amersham Biosciences Europe GmbH, Freiburg, Germany), in a total volume of 25 μL. One microlitre of the transcription/translation reaction was incubated in a total volume of 10 μL of 10 mM Hepes buffer, pH 7.2, supplemented with 150 mM potassium glutamate with scBoNT/D fusion proteins (3 nM final concentration) for 1 h at 37°C. Reactions were stopped by adding ice-cold fourfold concentrated sodium dodecyl sulfate sample buffer. Synaptobrevin and its cleavage products were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (15% gels), and radiolabelled protein was visualized using a BAS-1500 phosphor imager (Fuji Photo Film GmbH, Düsseldorf, Germany). The percentage of hydrolysed synaptobrevin was calculated applying the Tina 2.09f program (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

Mouse phrenic nerve toxicity assay

The MPN toxicity assay was performed as described previously (Habermann *et al*. 1980). The phrenic nerve was continuously stimulated at 5 V with a frequency of 1 Hz (0.1 ms pulse duration). Isometric contractions were transformed using a force transducer and recorded with the VitroDat Online software (FMI GmbH, Oberbeerbach, Germany). The time required to decrease the amplitude to 50% of the starting value (paralytic halftime) was determined. To compare the toxicity of scBoNT/D with that of the native BoNT/D, the naturally existing fully nicked form obtained from *Clostridium botulinum* cultures, the former was nicked between LC and HC.

The scBoNT/D was applied in triplicates at final concentrations of 1.2 pM, 4 pM, 12 pM, 40 pM and 120 pM, in order to compile a concentration–response curve to which the power function $y = 13.596x^{-0.2912}$ ($R^2 = 0.963$) could be ascribed. scBoNT/D fusion proteins were tested in duplicates at either 6.7 pM or 67 pM final concentrations. In some experiments, fusion proteins were applied in the presence of 0.6 μM Mtx. Resulting paralytic halftimes were converted to corresponding concentrations of scBoNT/D using the equation mentioned above, and toxicity was finally expressed as the percentage of the scBoNT/D toxicity.

Substrate cleavage in rat brain synaptosomes and primary cultures of mouse hippocampal neurones

Cleavage assays were conducted for 50 min at 37°C in duplicates in a total volume of 30 μL physiological buffer containing the individual proteins at final concentrations of 30 nM or 100 nM and 15 μL of synaptosome suspension. As control for the integrity of synaptosome preparations, incubation with the LC/D was run in parallel. After incubation, synaptosomes were lysed by the addition of ice-cold fourfold concentrated sample buffer containing 1 mM EDTA, and 10 μL aliquots were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Synaptobrevin 2 and SNAP-25 were detected following transfer onto nitrocellulose sheets using the monoclonal antibodies 69.1 (synaptobrevin 2) and 72.1 (SNAP-25) (Synaptic Systems GmbH, Göttingen, Germany), respectively, and secondary peroxidase coupled antibodies by enhanced chemiluminescence (Perbio Science GmbH, Bonn, Germany). Quantification was done using a Kodak image station 440cf applying the Kodak 1D v3.5.5SB program (Kodak). Substrate hydrolysis was calculated as the percentage reduction of the negative control immunoreactivity value.

Hippocampal neurones were prepared from embryonic day 17 mice as outlined earlier (Grosse *et al*. 2000). Neurones were plated on 35-mm cultures dishes at a density of $2 \times 10^5/cm^2$ and cultivated for up to 2 weeks. After 5 days *in vitro* (DIV), neurones were treated with BoNT/D, scBoNT/D, GFP-BoNT/D, or GFP-scBoNT/D for various periods as indicated, harvested and dissolved in sample buffer. Cleavage of synaptobrevin was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting.

Subcellular fractionation

Synaptosome preparations (30 μL aliquots) were incubated for 60 min at 0°C with luciferase or Luc-scBoNT/D each at 37 nM final

concentration in physiological buffer. Synaptosomes were then collected by centrifugation (4000 *g*), and supernatants were discarded. Pellet fractions were washed three times with 150 µL of ice-cold physiological buffer, then another 150 µL of buffer were added and the incubation was continued for 50 min at 37°C. Thereafter, synaptosomes were sedimented by centrifugation (4000 *g*), supernatants were discarded, and pellet fractions washed again three times. Before further processing, synaptosomes were treated with proteinase K (0.5 mg/mL final concentration) for 1 h on ice to remove non-internalized surface associated protein, and the reaction was stopped by the addition of phenylmethylsulfonyl fluoride (1.25 mM final concentration) in buffer supplemented with bovine serum albumin [3% (w/v)] and another incubation on ice for 15 min. Synaptosomes were then resuspended in 300 µL TC buffer (5 mM $CaCl_2$, 5 mM Tris-HCl pH 8.0, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 µM pepstatin A, 10 µM leupeptin, 300 mM saccharose, 5 mM benzamidine) subsequent to an additional sedimentation and washing cycle using 0.3% bovine serum albumin (w/v) in physiological buffer, and homogenized mechanically in a glass homogeniser. The resulting homogenate was centrifuged for 5 min at 4°C and 1000 *g*. The macrosomal pellet fraction (P1) was kept and the supernatant fraction was centrifuged for 90 min at 4°C and 125 000 *g* to yield the microsomal fraction (P2) and the cytosolic fraction (S2).

Luciferase assays

Aliquots of the various synaptosome fractions were replenished to a volume of 50 µL with extraction buffer [125 mM Tris-H_3PO_4, pH 7.8, 10 mM EDTA, 10 mM dithiothreitol, 50% glycerol, 5% Triton X-100 (v/v)]. After adding 300 µL of freshly prepared MP buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP), luciferase activity was measured using a Lumat LB9501 equipment (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) by injection of 100 µL luciferin solution (250 µM). Calibration curves for purified Luc-scBoNT/D and luciferase provided the basis for calculating the molar content of the respective protein in the various fractionation samples from measured relative light units. Values for luciferase served as controls for unspecific uptake and were subtracted from those of Luc-scBoNT/D.

Co-localization studies in chicken spinal cord cells

Chicken spinal cord cells (DIV 2; DIV 8 spinal cord cells were used for co-uptake studies with transferrin or dextran) were prepared according to Campenot *et al.* (1994) and incubated with nicked GFP-BoNT/D (57 nM final concentration) alone or in the presence of rhodamine-coupled human transferrin (30 mg/mL; Invitrogen GmbH, Karlsruhe, Germany) or rhodamine-conjugated dextran (Sigma-Aldrich; 1 mg/mL) for 10 or 30 min at 37°C. After washing with phosphate-buffered saline, cells were fixed for 10 min at 21°C in phosphate-buffered saline/4% *para*-formaldehyde and permeabilized during 5 min of incubation at 21°C in phosphate-buffered saline/4% *para*-formaldehyde, containing 1% Triton X-100 (v/v). Following washing with phosphate-buffered saline, incubation in phosphate-buffered saline/50 mM glycine and another washing step, cell preparations co-exposed to transferrin or dextran were immediately embedded for fluorescence microscopy, whereas cell preparations arranged for co-localization studies with lysobisphosphatidic acid rich domains were blocked in phosphate-buffered saline/10% fetal calf serum/0.5% bovine serum albumin and then incubated with 6C4 antiserum at a 1 : 100 dilution for 1 h at 21°C in phosphate-buffered saline/0.5% fetal calf serum/0.5% bovine serum albumin. Antigen was subsequently decorated after interim threefold washing with phosphate-buffered saline by rhodamine-coupled anti-mouse IgG antibody (1 : 50 dilution; Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 21°C in phosphate-buffered saline/0.5% fetal calf serum/0.5% bovine serum albumin. Cells were finally washed three times and embedded in 0.13 M Tris-HCl, pH 8.5 supplemented with Mowiol [13% (w/v)], 1,4-diazabicyclo[2.2.2]octane [33% (w/v)] and glycerol [33% (w/v)], and antigens were visualized by fluorescence microscopy.

Data analysis

All results are presented as mean ± SD. Differences between results were tested by analysis of variance (ANOVA) with Bonferroni post test or Student's *t*-test, respectively, using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, USA, http://www.graphpad.com). A probability of $p < 0.05$ was considered significant.

Results

Production, purification and characterization of botulinum neurotoxin type D fusion proteins

The full-length BoNT/D was generated in *E. coli* under biosafety level 2 containment (project approved by the district administration Hannover, Az 501g.40654/3/57/3) resulting in a ~150 kDa single chain protein with a yield of 3–4 mg/L bacteria culture. This single chain form (denoted scBoNT/D throughout) could be effectively nicked by immobilized trypsin generating the corresponding HC and LC (Fig. 1b) that remained connected by the interchain disulphide bridge under non-reducing conditions (denoted BoNT/D throughout; data not shown). BoNT/D fusion proteins with DHFR, GFP, LC/A and luciferase (Fig. 1a) were produced as single chain proteins as well, exhibiting the expected sizes of approximately 171 kDa, 176 kDa, 200 kDa and 212 kDa, respectively (Fig. 1b). Protein yields, however, ranged between 12% [LC/A-scBoNT/D(E230A)] and 85% (DHFR-scBoNT/D) compared to the scBoNT/D. Trypsin treatment selectively nicked all fusion proteins between HC and LC without liberating their cargo proteins (Fig. 1b). Interchain disulphide bridges were formed (data not shown). In keeping with earlier findings (Thompson *et al.* 1991, 1997; Klingenberg and Olsnes 1996), use of larger amounts of trypsin resulted in concomitant degradation of the DHFR and the Luc portion of these fusion proteins. Pre-incubation of DHFR-scBoNT/D with the folate analogue Mtx drastically reduced the sensitivity towards trypsin (data not shown), evidencing correct folding of the DHFR portion (Klingenberg and Olsnes 1996).

To ensure that fusions to BoNT/D did not change the ability of the molecules to bind to neuronal membranes, their interaction with synaptosomes was assessed. Approximately

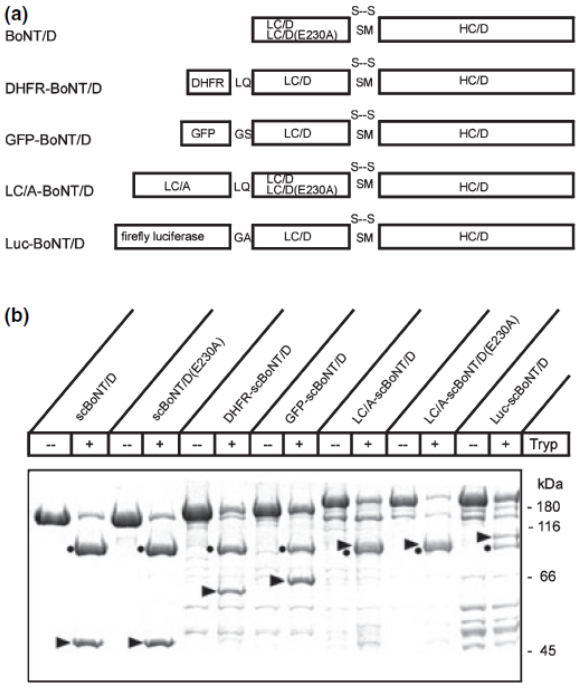

Fig. 1 Survey of generated botulinum neurotoxin type D (BoNT/D) fusion proteins. (a) Schematic representation of the fusion proteins. S—S denotes the interchain disulphide bridge formed between light chain (LC) and heavy chain (HC) of BoNT/D. Letters between bars specify amino acids in the single letter code that were chosen as linkers between fusion protein segments. (b) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of the various BoNT/D fusion proteins after single step purification on StrepTactin-sepharose beads and, where indicated, treatment with trypsin (Tryp; 1 : 4000 w/w for 40 min at 21°C). Approximately 1.5 μg of each protein was loaded and stained by Coomassie blue employing 10% gels run under reducing conditions. All fusion protein preparations proved to be approximately 90% pure as determined by densitometric scanning. HC/D formed on trypsin treatment is marked with black dots. Cargo protein-LC/D portions are labelled with arrowheads. Importantly, trypsin treatment did not liberate cargo proteins from LC/D.

1 μg recombinant protein was incubated with synaptosomal preparations for 1 h at 0°C. After intensive washing, synaptosomes were solubilized and bound protein was detected following sodium dodecyl sulphate–polyacrylamide gel electrophoresis and protein transfer onto nitrocellulose by enhanced chemiluminescence employing a StrepTag specific antiserum. Binding affinities of the fusion proteins were not altered compared to scBoNT/D and binding of DHFR-scBoNT/D was not affected in the presence of 0.6 μM Mtx (Table 1). Likewise, the catalytic activity of the various scBoNT/D fusion proteins was studied *in vitro* at 3 nM final concentrations of fusion proteins using rat synaptobrevin 2 as substrate (Yamasaki *et al.* 1994). No significant change in the catalytic activity was determined in comparison to scBoNT/D, as well as in the presence of 0.6 μM Mtx (Table 1).

Table 1 Binding and catalytic properties of single chain botulinum neurotoxin type D (scBoNT/D) and its fusion proteins

BoNT	Binding[a] (% of scBoNT/D)	Catalytic activity[b] (% cleavage of synaptobrevin 2)
scBoNT/D	100 ± 4	60.2 ± 5.2
DHFR-scBoNT/D	98 ± 7	67.3 ± 3.8
DHFR-scBoNT/D + Mtx[c]	99 ± 2	63.7 ± 7.2
GFP-scBoNT/D	99 ± 5	76.1 ± 3.2
LC/A-scBoNT/D	103 ± 6	66.5 ± 12.6
LC/A-scBoNT/D(E230A)	102 ± 3	b.d.l.[d]
Luc-scBoNT/D	99 ± 4	66.6 ± 11.3

[a]Binding was analysed using rat brain synaptosomes. Amounts of bound fusion proteins were determined via immunoblot and are depicted as percentage of the value for scBoNT/D that was set to 100%. Data represent means ± SD ($n = 4$).
[b]The catalytic activity of scBoNT/D fusion proteins (3 nM final concentration) was tested *in vitro* employing ^{35}S-labelled BoNT/D substrate synaptobrevin 2. The extent of substrate hydrolysis was quantified via phosphor imaging. Data represent means ± SD ($n = 4$).
[c]Mtx: 0.6 μM.
[d]b.d.l., below detection limit.
DHFR, dihydrofolate reductase; GFP, green fluorescent protein; LC/A, light chain of BoNT/A; Luc, luciferase.

Determination of the toxicity of botulinum neurotoxin type D fusion proteins at the mouse phrenic nerve

To assess the influence of the various cargo proteins on the ability of the BoNT/D to bind to motor nerve endings, to undergo internalization and translocation, as well as to subsequently block neurotransmitter release, their activity was determined employing the MPN toxicity assay. scBoNT/D displayed 10% toxicity compared to the native BoNT/D, whereas recombinant BoNT/D proved to be equally potent (Fig. 2a). Fusion proteins were applied as single chain proteins. Therefore, their toxicity was calculated basing on the dose–response curve of the scBoNT/D and finally expressed as the percentage of the scBoNT/D toxicity (Fig. 2b). Attachment of DHFR did not reduce the activity. The fusion with luciferase generated a molecule with approximately 7% residual toxicity, whereas the addition of the smaller GFP diminished the toxicity even more. Interestingly, LC/A-scBoNT/D proved to be slightly more potent than scBoNT/D. This probably originates from the simultaneous proteolytic attack of the two catalytically active LCs, i.e. of LC/A and of LC/D, on the transiently accessible pools of synaptobrevin and SNAP-25 during stimulation yielding independently impairment of the vesicular fusion machinery. In line with this interpretation, LC/A-scBoNT/D(E230A), which contains only the enzymatically active LC/A, exhibited merely 24 ± 2.1% toxicity. Additionally, the remaining activity of LC/A-scBoNT/D(E230A) clearly proves that the cargo protein LC/A is operative after translocation.

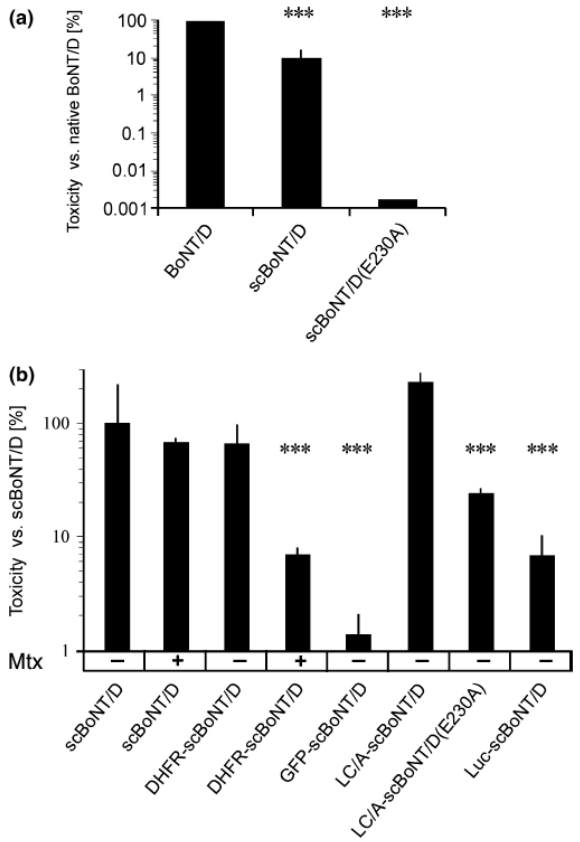

Fig. 2 Toxicity of botulinum neurotoxin type D (BoNT/D), single chain BoNT/D (scBoNT/D), various scBoNT/D-derived fusion proteins in the mouse phrenic nerve toxicity assay. Note the logarithmic scale of the abscissas. (a) The toxicity of BoNT/D, scBoNT/D and scBoNT/D(E230A) was determined and expressed as the percentage toxicity of the native BoNT/D, which naturally exists in the di-chain form. (b) The toxicity of various scBoNT/D fusion proteins was determined vs. the scBoNT/D. The toxicity of scBoNT/D and dihydrofolate reductase-scBoNT/D (DHFR-scBoNT/D) was also analysed in the presence of methotrexate (Mtx: 0.6 μM final concentration). Data represent mean values ± SD of two to four independent experiments performed in duplicates. Statistically significant differences of toxicities vs. native BoNT/D (a) or scBoNT/D (b): ***$p < 0.001$.

Pre-incubation of DHFR-scBoNT/D with Mtx resulted in a strong reduction of toxicity by a factor of approximately 10. Mtx stabilizes the native conformation of DHFR, thereby obstructing unfolding during the translocation processes (Eilers and Schatz 1986). Mtx had a minor but statistically not significant effect on the toxicity of scBoNT/D. The drastic effect of Mtx on the toxicity of DHFR-scBoNT/D and the lack of correlation between toxicity and the size of the attached cargo protein argue for the necessity of partial unfolding of the protein to be delivered before or as one step during translocation from the lumen of endocytic vesicles to the cytosol.

Determination of the proteolytic activity of botulinum neurotoxin type D fusion proteins at synaptosomes and primary cultures of mouse hippocampal neurones

Although the MPN assay provided information about the physiological toxicity of the various fusion proteins, the use of synaptosomes or cultured neurones permits direct visualization of the ability of the individual proteins to cleave their cytosol exposed substrate molecule. Aliquots of synaptosomal preparations exhibiting a protein content of 150 μg were incubated with single chain fusion proteins at a final concentration of 30 nM in a total volume of 30 μL for 50 min at 37°C. The BoNT/D hydrolysed 89 ± 6% of its substrate synaptobrevin 2 under these conditions (data not shown) and was used as a reference. Cleavage products of synaptobrevin 2 were not detectable, most likely due to an enhanced susceptibility towards further degradation (Link *et al.* 1992). As presented in Fig. 3, fusion of BoNT/D with DHFR or LC/A had little effect on the endoprotease activity of LC/D, whereas the attachment of luciferase or GFP diminished

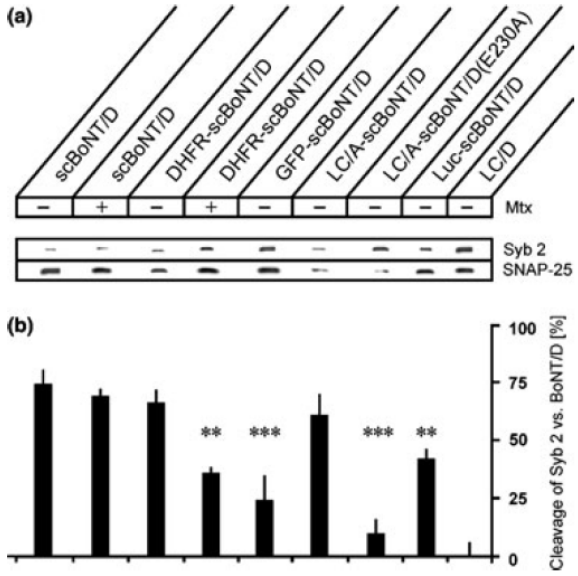

Fig. 3 Proteolytic activity of various single chain botulinum neurotoxin type D (scBoNT/D) fusion proteins in rat brain synaptosomes. (a) Synaptosomes were incubated for 50 min with scBoNT/D or various scBoNT/D fusion proteins (30 nM) at 37°C. The proteolytic activity of scBoNT/D and dihydrofolate reductase-scBoNT/D (DHFR-scBoNT/D) was also analysed in the presence of Mtx (0.6 μM final concentration). Substrate was detected by western blotting via monoclonal antibodies and enhanced chemiluminescence following sodium dodecyl sulphate–polyacrylamide gel electrophoresis. (b) Quantification of substrate hydrolysis was done employing a Kodak image station and the Kodak 1D version 3.5.5SB program. Values are depicted as the percentage of abolished substrate vs. control samples treated with BoNT/D. Data represent mean values ± SD of two to three independent experiments performed in duplicates. Statistically significant differences of proteolytic activities vs. scBoNT/D: **$p < 0.01$; ***$p < 0.001$.

substrate hydrolysis by approximate factors of 2 and 3, respectively. These results largely match those obtained in the MPN assay, but the effects were in general less pronounced. A possible explanation might be a higher availability of surface receptors in synaptosomes. Again, pre-incubation of DHFR-scBoNT/D with Mtx significantly reduced the substrate cleavage rate in synaptosomes, pinpointing that a stabilized conformation of DHFR hampers entry of the LC into the cytosol. Notably, for the first time, our study directly proves that cargo enzymes develop their enzymatic activity after CNT mediated delivery to the cytosol, as SNAP-25 was efficiently hydrolysed by fusion proteins LC/A-scBoNT/D and LC/A-scBoNT/D(E230A) (44 ± 8.6% and 45 ± 6.0% substrate cleavage, respectively; Fig. 3a).

Next, we assessed the efficacy of BoNT/D loaded with GFP in long-term experiments, as GFP-scBoNT/D represents the least active and least protease sensitive fusion protein (Fig. 2b; data not shown), the latter a requirement for such long-term experiments. Starting at DIV 5, mouse hippocampal neurones were cultured in the presence of the neurotoxins (100 pM each) and the amount of synaptobrevin in cell lysates was determined following sodium dodecyl sulphate–polyacrylamide gel electrophoresis by immunostaining 1, 2, 3 and 6 days later. A comparison with BoNT/D or scBoNT/D shows that GFP-BoNT/D and GFP-scBoNT/D were capable of effectively hydrolysing synaptobrevin in the course of 6 days of incubation (Fig. 4). Thus, the reduced enzymatic activity observed for GFP-scBoNT/D at the MPN and synaptosomes could be overcome by prolonging the incubation time. This suggests that the translocation process is not generally impaired but prolonged due to the attachment of this particular cargo.

Detection of luciferase translocation

Encouraged by these findings, we investigated whether the enzymatic activity of non-LC cargo proteins would be detectable in the cytosol. Synaptosomes were therefore incubated with Luc-scBoNT/D or luciferase as a negative control (each 7.1 pmole) at 0°C. After removal of unspecifically bound protein by intensive washing, endocytosis was triggered by a temperature rise to 37°C. Subsequent to 50 min of incubation, surface attached protein was removed by proteinase K treatment and synaptosomes were then fractionated into macrosomal (P1), microsomal (P2) and cytosolic fractions (S2). As shown in Fig. 5, approximately 1% of the total synaptosome associated luciferase activity of Luc-scBoNT/D was recovered in the microsomal fraction, whereas approximately 0.4% was detected in the cytosol. Thus, the attachment of luciferase to BoNT/D warranted the entry of significant amounts of this enzyme into the cytosol.

An analogous experiment with DHFR-scBoNT/D failed due to the much less sensitive detection system for DHFR, i.e. the photometric assay recording the turnover of NADPH

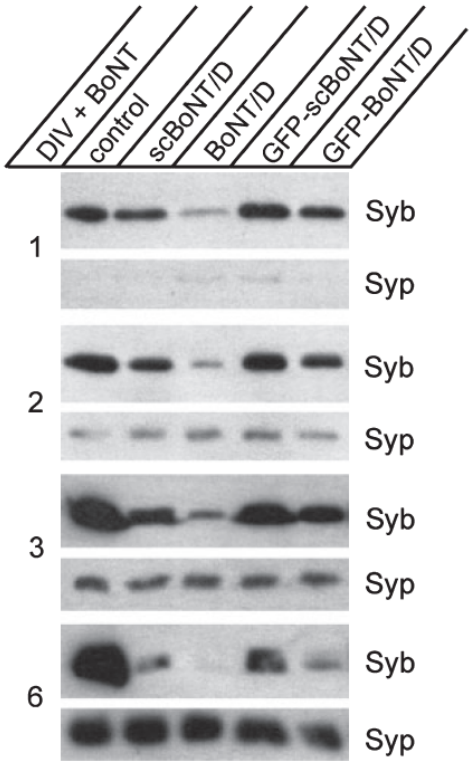

Fig. 4 Time course of synaptobrevin cleavage by green fluorescent protein-botulinum neurotoxin type D (GFP-BoNT/D) in hippocampal neurones. Mouse hippocampal neurones (prepared at embryonic day 17) were treated at days *in vitro* (DIV) 5 with 100 pM of single chain BoNT/D (scBoNT/D), BoNT/D, GFP-scBoNT/D and GFP-BoNT/D. Neurones were harvested at the indicated time, dissolved in sample buffer and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting using monoclonal antibodies against synaptobrevin 2 (Syb). Immunostaining of synaptophysin (Syp) served as control for equal load of protein. All BoNT/D preparations cleaved synaptobrevin with, as expected, the nicked preparations being more efficient. Notably, GFP-BoNT/D and GFP-scBoNT/D approached the efficacy of BoNT/D and scBoNT/D, respectively, after prolonged incubation time. As expected, the amount of synaptophysin and synaptobrevin increased during cultivation time.

to $NADP^+$ ($NADPH + H_2\text{-folate} + H^+ \rightarrow NADP^+ + H_4\text{-folate}$). DHFR could merely be detected in the synaptosome associated material and the macrosomal fraction, but not in microsomal and cytosolic samples (data not shown).

Uptake route of green fluorescent protein-botulinum neurotoxin type D

In order to check whether the attachment of cargo protein affects the documented uptake route of BoNTs via receptor mediated endocytosis and acidic compartments, the entry of GFP-BoNT/D, which exhibited the least activity in both the MPN and the synaptosomal assay, was studied by two different techniques in cultured mouse hippocampal neurones

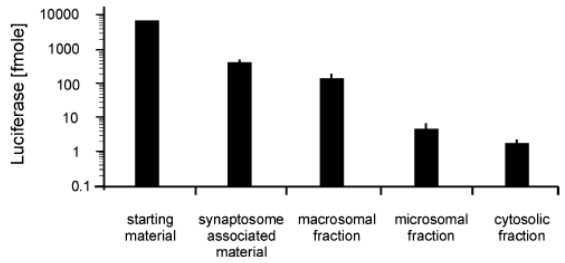

Fig. 5 Detection of luciferase activity in subcellular fractions. Luciferase activity was determined by bioluminescence employing a Lumat LB9501 equipment. Amounts of Luc-scBoNT/D and firefly luciferase associated with the various fractions, the latter ones serving as controls for unspecific uptake, were calculated via calibration curves from relative light units. Control values of luciferase were subtracted from those of Luc-scBoNT/D. Data represent mean values ± SD of four independent experiments. Approximately 0.4% of the total synaptosome associated luciferase activity of Luc-scBoNT/D was detected in the cytosol.

and chicken spinal cord cells. Baf A1, a drug blocking the activity of vesicular H^+-ATPases and consequently the acidification of endocytic vesicles, inhibited cleavage of synaptobrevin 2 by BoNT/D in cultured mouse hippocampal neurones (Fig. 6a; Williamson and Neale 1994; Schoonderwoert *et al.* 2000). As substrate cleavage by GFP-BoNT/D was reduced to a comparable extent by incubation with Baf A1, attachment of GFP does not appear to affect the transport route of BoNT/D through acidic compartments. Concordant results were obtained in assays using synaptosomes (data not shown).

In chicken spinal cord cells we conducted co-localization experiments. GFP fluorescence enabled a direct visualization of GFP-BoNT/D in cellular compartments. It largely co-localized with the simultaneously applied rhodamine-coupled transferrin, a marker for receptor-mediated endocytosis, 10 and 30 min after their addition (data not shown and Fig. 6b, respectively). In line with these results, GFP-BoNT/D also co-localized with material recognized by the lysobisphosphatidic acid rich domains specific antibody 6C4 (Fig. 6b; Kobayashi *et al.* 2001). In addition, the lack of co-localization with rhodamine-coupled dextran (Fig. 6b) proves that uptake does not occur via fluid phase endocytosis and further corroborates an uptake route that passes through acidic compartments.

Discussion

In the present study we assessed whether cargo protein attached by gene fusion to proteolytically inactive full-length BoNT could represent a suitable transport system for the delivery of the cargo portion to the cytosol of neuronal cells. This approach is superior to earlier chemical conjugation strategies due to the prevention of undefined side-products that have subsequently to be removed or low yield of the desired linkage product, provided the fusion protein can efficiently be produced. Satisfying production of fusion proteins indeed succeeded, albeit the yield decreased upon the attachment of the cargo protein compared to BoNT/D.

The attachment of cargo proteins did not impair binding abilities of BoNT/D. Also its uptake route did not differ from established data, i.e. entry into neurones via recycling vesicles and transport through an acidic compartment, as the fusion proteins co-localized with transferrin, showed partial co-localization with lysobisphosphatidic acid-rich membrane domain organelles (mAb 6C4), and translocation was sensitive to Baf A1. Thus, fusion proteins such as GFP-BoNT/D may represent valuable tools to study the intracellular transport of CNTs, especially when pH sensing variants of GFP would be used. Alterations of their biological activity via chemical attachment of fluorophors and deviation from their natural trafficking route, i.e. retention in vesicular compartments, owing to the lack of the translocation domain or the LC are at least clearly excluded. As earlier suggested by Li *et al.* (2001), corresponding fusion proteins with TeNT are of particular interest, since TeNT is differently sorted subsequent to receptor mediated endocytosis to undergo retrograde axonal transport (Price *et al.* 1975), to traverse the synaptic cleft, and to act in inhibitory neurones of the spinal cord. In our hands, for example, Luc-scTeNT and scTeNT-GFP could also be produced with acceptable yield (data not shown).

Following receptor-mediated endocytosis, CNTs reach low pH vesicular compartments. Low pH is assumed to trigger a conformational change, exposition of hydrophobic regions within the H_N-domain (Boquet *et al.* 1984) and their insertion into vesicular membranes (Roa and Boquet 1985). Pore formation allows the catalytic subunit to translocate into the cytosol. The LC itself was shown to undergo structural changes at low pH, forming a molten globule structure (Li and Singh 2000) and recent data of Koriazova and Montal (2003) provided evidence that translocation through reconstituted lipid bilayers occurs with a partially unfolded LC conformation, whereupon the HC presumably functions as a pH-driven transmembrane chaperone. Our data are in entire compliance with this conclusion. In the absence of Mtx, DHFR-scBoNT/D exhibited virtually identical neuroparalytic activity as scBoNT/D. Application of Mtx, however, which stabilizes the conformation of DHFR (Eilers and Schatz 1986), revealed that unfolding of the cargo and LC is also requisite for translocation in nerve cell systems, as evidenced by the roughly 10-fold less efficient transport of DHFR-LCD. This finding together with the fact that the measured ~15 Å diameter pore size for BoNT/A (Koriazova and Montal 2003) is too narrow to permit passage of the 55 Å × 55 Å × 62 Å-sized BoNT/A LC, implies that the LC itself must also unfold during the membrane penetration process. In this respect, the membrane penetration process of BoNT LCs resembles that of other di-chain protein toxins, as

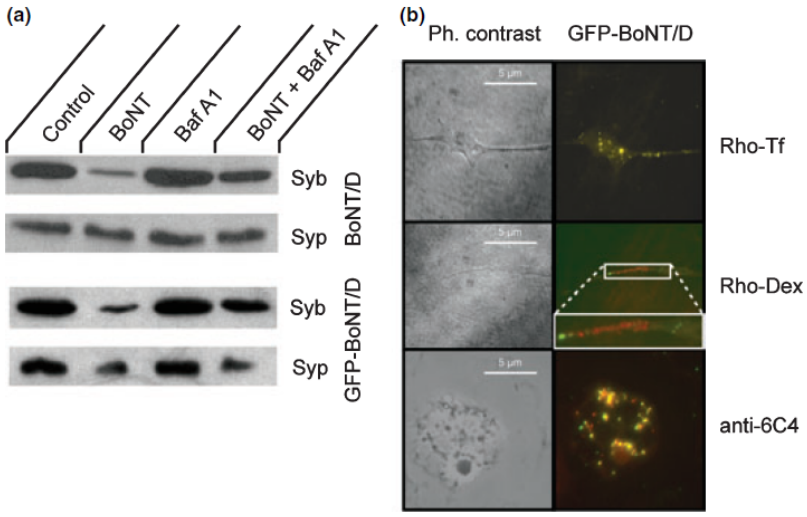

Fig. 6 Analysis of the uptake route of botulinum neurotoxin type D (BoNT/D) and green fluorescent protein-BoNT/D (GFP-BoNT/D). (a) Mouse hippocampal neurones were treated at days *in vitro* (DIV) 8 for 15 min with 10 nM Bafilomycin A1 (Baf A1) at 37°C followed by 4 h of incubation with 10 nM of BoNT/D or GFP-BoNT/D. Synaptobrevin 2 (Syb) and synaptophysin (Syp), the latter serving as control for the amount of loaded protein, were detected by western blotting via monoclonal antibodies and enhanced chemiluminescence. Baf A1 pre-treatment drastically decreased cleavage of synaptobrevin 2 by BoNT/D and GFP-BoNT/D evidencing that both proteins pass through acidic compartments en route delivering the catalytic domain to the cytosol. (b) GFP-BoNT/D at a final concentration of 57 nM was incubated in the presence of rhodamine-coupled transferrin (Rho-Tf; 30 mg/mL), rhodamine-conjugated dextran (Rho-Dex; 1 mg/mL) or alone with chicken spinal cord cells (DIV 2–8) for 30 min at 37°C. After washing, the anti-6C4 antibody was used to specifically decorate early endosomes in cells previously incubated with GFP-BoNT/D alone (anti-6C4). Note that GFP-BoNT/D co-localizes with transferrin (yellow spots), but not with dextran, and passes through the early endosomal compartment as evidenced by its partial co-localization with 6C4 decorated organelles (yellow spots). Left panels show phase contrast images of the same sections illustrated on the right. The inset in the Rho-Dex panel shows a higher magnification of the boxed section. Taken together, attachment of GFP does not affect the uptake route of BoNT/D.

fusion of DHFR to the catalytic domains of ricin or diphtheria toxin also strictly depended on a non-stabilized conformation of DHFR (Klingenberg and Olsnes 1996; Beaumelle *et al.* 1997). Similarly, translocation of the diphtheria toxin catalytic domain was impeded when the tight conformation of attached fibroblast growth factor was induced by the addition of heparin, inositol hexaphosphate or inorganic sulphate (Wiedlocha *et al.* 1992).

In the present work, we directly prove for the first time that cargo proteins attached to CNTs regain their enzymatic activity after exiting the endosome, as demonstrated by the cleavage of SNAP-25 in synaptosomes by the attached BoNT/A LC and accordingly the detection of luciferase activity of the firefly luciferase-BoNT/D fusion protein in synaptosomal cytosol preparations. Thus, these findings reveal that CNT HCs can act as transmembrane transport facility for cargo proteins that are hooked to the LC. The capacity of the CNT translocation machinery was characterized by means of attaching various cargo proteins featuring different sizes and structures. It turned out that the size of the cargo protein is not the sole determinant for efficient delivery to the cytosol. The efficacy decreased with increasing size of the passenger protein [compare toxicities of DHFR-scBoNT/D, LC/A-scBoNT/D(E230A) and Luc-scBoNT/D, Fig. 2b], but structural restraints of the attached passenger protein are apparently the more critical parameter. That is to say, GFP, although representing the smallest cargo protein tested, was transported with the least efficiency. The neuroparalytic activity of GFP-scBoNT/D in the MPN assay was 50-fold lower compared with that of the similar sized DHFR-scBoNT/D, and also substantially lower compared to Luc-scBoNT/D, whose cargo portion exhibits more than twice the size. The β-barrel-shaped structure of GFP (Brejc *et al.* 1997; Palm *et al.* 1997) seems to slow down translocation even stronger than does the conformationally stabilized DHFR-Mtx. In agreement with it, GFP represents an extremely stable protein that did not unfold at temperatures up to 85°C at physiological pH (7.4) and remained stable up to 70°C at low pH (5.0) as assessed by circular dichroism spectroscopy (A. Rummel, T. Binz and J. Alves, unpublished results). However, alternative explanations for inefficient translocation of cargo proteins are conceivable, among them a lack of interaction with cellular chaperones such as the recently characterized complex for the translocation of the diphtheria toxin catalytic domain (Ratts *et al.* 2003) or an inability of adopting a molten globule-like conformation at acidic pH

that was recognized as a trigger for forming the transmembrane conformation in case of diphtheria toxin (Ren *et al.* 1999). Additional experiments employing further cargo proteins will ultimately define the selectivity of the BoNT translocation machinery. So far, our data support a versatile usability of BoNT/D.

An obvious potential pharmaceutical application for BoNT/D may be linked to the use of BoNT/A and BoNT/B as approved drugs for the treatment of certain neurological diseases (Münchau and Bhatia 2000). These serotypes are superior to other serotypes due to the so far unsettled longevity of their effects, lasting for several months. Not uncommonly, although administered merely in nanogram quantities, patients develop antibodies against the neurotoxins, which prohibits further application of the respective BoNT serotype. In this study we presented the effective transportation of LC/A into the cytoplasm of neurones using BoNT/D as transport vehicle. This finding opens the opportunity to replace native BoNT/A and BoNT/B as remedies for patients who established immunity against BoNT/A and B. Because the H_C-fragment is the dominant immunogenic portion of the molecule (Turton *et al.* 2002), fusion proteins consisting of the LC of serotype A or B and full-length BoNT/D, E, F, or G would presumably still allow to deliver a 'persistent' LC by bypassing the immuno response anti BoNT/A and B.

Recently, the translocation domain of BoNT/A was shown to function independently from the cell binding domain, such that the molecule could be re-targeted to penetrate into cells others than cholinergic neurones. Replacement with nerve growth factor (Chaddock *et al.* 2000a), wheat germ agglutinin (Chaddock *et al.* 2000b) or *Erythrina cristagalli* lectin (Duggan *et al.* 2002) allowed import of LC/A into PC12 cells, pancreatic β-cells and nociceptive afferents, respectively. Thus, a future goal could consist in designing proteolytically inactive BoNT fusion proteins with replaced cell binding domains to transport cargo proteins that complement missing enzymatic activities in predetermined tissue.

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“The C-terminal transmembrane region of synaptobrevin binds synaptophysin from adult vesicles.”

Yelamanchili SV, Reisinger C, Becher A, Sikorra S, Bigalke H, Binz T, Ahnert-Hilger G.

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Erklärung über den Anteil an den Publikationen

The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis. - Reisinger C, Yelamanchili SV, Hinz B, Mitter D, Becher A, Bigalke H, Ahnert-Hilger G.

Zwei Drittel dieser Publikation entstammen eigener Arbeiten, diese sind im Folgenden aufgeführt:

Der Nachweis der konzentrationsabhängigen Wirkung von Botulinumtoxin A wurde in hippocampalen Neuronkulturen bei Applikation von 0,1pM bis 100pM Toxin erbracht. Die Auswertung erfolgte nach Sedimentierung der Neurone und Auftrennung der Proteine über SDS-Page und Westernblot sowie durch Immundetektion mit Antikörper gegen SNAP25 und gegen die durch Spaltung freigelegte Sequenz am Ende von SNAP25 (Antikörper p1-16). Die proteolytische Abspaltung von 9 Aminosäuren ist erkennbar am Shift (erhöhte Wanderungsgeschwindigkeit des gekürzten SNAP25) und über den Antikörper p1-16. Die Versuche führten zu den Abbildungen 2a und zeigen, dass die Nachweisgrenze der Wirksamkeit von BoNT/A nach 8 Tagen Inkubation bei 2pM liegt.

Des Weiteren wurde in Synaptosomen die Wirkung von BoNT/A in Konzentrationen von 20nM bis 100nM getestet. Dies wurde einerseits nativ und andererseits analog zu dem in Abbildung 1b gezeigten Versuch mit durch Dithiotreitol (DTT) präaktiviertem Toxin in mit SLO-permeabilisierten Synaptosomen durchgeführt. (Ergebnisse nicht gezeigt). Hier zeigte sich, dass die minimale Konzentration für die proteolytische Spaltung von SNAP 25 durch BoNT/A in Synaptosomen nach 2h in beiden Ansätzen 100nM beträgt.

Der neu eingeführte Antikörper p1-16 wurde in mehreren Versuchen evaluiert und zeigte sich zu 100% spezifisch (kein Signal bei intaktem SNAP25).

Die Immunpräzipitation über den Antikörper gegen Synaptobrevin von mit BoNT/A inkubierten hippocampalen Neuronen führte zur Abbildung 2b. Hierbei fand sich keine quantitative Änderung im Synaptophysin/Synaptobrevin-Komplex. Zusätzlich wird die unveränderte Bindung von dem um 9 Aminosäuren gekürzten SNAP25 an Synaptobrevin dargestellt.

Die Behandlung von hippocampalen Neuronen mit BoNT/A in Gegenwart und Abwesenheit von LTX und die anschließende Auswertung über Immunpräzipitation führte zu den Abbildungen 3c. Bei gleichem Versuchsaufbau und nach Stimulation mit einer Ca2+-Ionophore entstand Abbildung 4. Diese Versuche zeigen, dass kurzzeitige Stimulationen zu einer Abnahme des Synaptophysin/Synaptobrevin-Komplexes führen, welche nicht durch BoNT/A Behandlung antagonisiert werden kann und somit unabhängig von der Membranfusion ist.

The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content. - Mitter D, Reisinger C, Hinz B, Hollmann S, Yelamanchili SV, Treiber-Held S, Ohm TG, Herrmann A, Ahnert-Hilger G.

Diese Arbeit beruht zu etwa einem Viertel auf eigenen Resultaten:

Das Ergebnis nach Zugabe von in Ethanol gelöstem Cholesterol zu hippocampalen Neuronen in der Wachstumsphase und die anschließende Darstellung des Synaptophysin/Synaptobrevin-Komplexes über Immunpräzipitation ist in Abbildung 5b und c gezeigt. Hier zeigt sich eine signifikante Zunahme in der Interaktion der beiden Proteine im Vergleich zu Kontrolle.

In nicht gezeigten Studien wurde die Bindung von Synaptobrevin zu Synaptoporin nach Cholesterolzugabe geprüft. Es wurden außerdem eigene Versuche zur Depletion von Cholesterol mit β-MCD in hippocampalen Neuronen durchgeführt, die zur selben Schlussfolgerung wie die in den Abbildungen 2 gezeigten Versuche führten. Damit konnte nach Entzug von Cholesterol eine Abnahme der Komplexbildung nachgewiesen werden wohingegen die Zugabe eine Erhöhung der Komplexbildung bewirkte.

The C-terminal transmembrane region of synaptobrevin binds synaptophysin from adult vesicles. - Yelamanchili SV, Reisinger C, Becher A, Sikorra S, Bigalke H, Binz T, Ahnert-Hilger G.

Ca. ein Drittel dieser Veröffentlichung konnte in eigenen Versuchen dargestellt werden, diese sind im Einzelnen:

In eigenen Vorarbeiten wurde auf der Basis eines bereits vorhandenen Synaptobrevin-Klons mit N-terminalem HisTag über Einklonierung der vorhandenen DNA in einen neuen Vektor, ein Synaptobrevin mit C-terminalem HisTag hergestellt, der in den gezeigten Versuchen jedoch keine Verwendung fand.

Sowohl N- als auch C-terminal getagtes Synaptobrevin wurden an Nickelbeads gebunden, wahlweise über TeNt gespalten und mit Extrakt aus Vesikelmembranen inkubiert. Die Auswertung der Eluate ist in Abbildung 3a zu sehen. Weitere Versuche zeigten mit Abbildung 4b übereinstimmende Ergebnisse in Bezug auf Syntaxin und SNAP25. Insgesamt konnte gezeigt werden, dass komplettes Synaptobrevin mit HisTag wie erwartet sowohl SNAP25 und Syntaxin als auch Synaptophysin bindet und dass einerseits die N-terminale Hälfte nur noch SNAP25 und Syntaxin bindet, während andererseits die C-terminale Hälfte nur Synaptophysin bindet.

Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. - Bade S, Rummel A, Reisinger C, Karnath T, Ahnert-Hilger G, Bigalke H, Binz T.

Einen Anteil von etwa einem Fünftel konnte ich zu dieser Publikation beitragen:

Der Vergleich von verschiedenen BoNT/D-Konstrukten in hippocampalen Neuronen ist in Abhängigkeit von der Wirkdauer in Abbildung 4 dargestellt. Zusätzlich wurde in nicht gezeigten Versuchen die Konzentrationsabhängigkeit beschrieben. Hier zeigte sich, dass mit einem Cargoprotein gekoppeltes Toxin zwar schwächer aber durchaus wirksam ist.

In ähnlichen Ansätzen war die BoNT-Wirkung über eine Hemmung der vesikulären H+-ATPase durch Bafilomycin antagonisierbar, was in Abbildung 6 zu sehen ist. Diese Experimente zeigen, dass die Verlagerung der katalytischen Domäne ins Zytosol durch saure Kompartimente, sowohl bei dem nativem als auch dem Fusionsprotein geschieht.

Eidesstattliche Erklärung

Ich, Clemens Reisinger, erkläre an Eides statt, dass ich die vorgelegte Dissertationsschrift mit dem Thema: “Regulation der Interaktion der präsynaptischen Vesikelproteine Synaptophysin und Synaptobrevin“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Lebenslauf

persönliche Angaben

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